Bacterial cells with improved tolerance to diacids

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(54) Title: BACTERIAL CELLS WITH IMPROVED TOLERANCE TO DIACIDS

(57) Abstract: The present invention relates to bacterial cells genetically modified to improve their tolerance to certain commodity chemicals, such as diacids, and to methods of preparing and using such bacterial cells for production of diacids and other compounds.

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BACTERIAL CELLS WITH IMPROVED TOLERANCE TO DIACIDS

FIELD OF THE INVENTION

The present invention relates to bacterial cells genetically modified to improve their tolerance to certain commodity chemicals, such as dicarboxylic acids (herein referred to as "diacids") and other polycarboxylic acids, and to methods of preparing and using such bacterial cells for production of diacids and other compounds.

BACKGROUND OF THE INVENTION

Aliphatic diacids are commonly used as precursors for nylon polymers (polyamides), typically prepared by condensing diamines with diacids. Diacids are also used as monomers for various other polymers and copolymers including polyurethanes. Different chain lengths and the presence of unsaturated bonds or branched chains within the constituent diacids imparts different physical properties to the polymer.

There has been significant recent interest in producing diacids biologically, *i.e.*, in microbial cells. For example, as reported on their respective websites (accessed in October 2016), Myriant Corporation and BioAmber Inc. have both begun biological production of succinic acid, as a replacement molecule for petrochemical-derived adipic acid, Verdezyne Inc. is developing a process to produce adipic acid in yeast, and one of the major worldwide manufacturers of nylons, INVISTA™, is actively seeking the development of biologically produced precursors through collaborations with external parties. The production of diacids in metabolically engineered microbial cells have been reviewed and described in several publications such as, *e.g.*, Polen *et al.*, 2013; Adkins *et al.*, 2013; Park *et al.*, 2013; Yu *et al.*, 2014; Cheong *et al.*, 2016; Deng and Mao, 2015; WO 2011/003034 A2 (Verdezyne); Curran *et al.*, 2013; Sengupta *et al.*, 2015; and Zhang *et al.*, 2015.

For production of bulk chemicals from renewable plant-based carbon feedstocks, high product titers are essential in order to minimize capital equipment and downstream separations costs for product purification. At the high titers required for economical fermentation processes, however, most chemicals exhibit significant toxicity that reduces yields and productivities by negatively affecting microbial growth (Van Dien, 2013; Zingaro *et al.*, 2013).

Escherichia coli being a suitable host for industrial applications, there has been some interest in developing *E. coli* strains with improved tolerance to chemicals of interest for production, such as, *e.g.*, n-butanol, ethanol and isobutanol, or to stress conditions present during

fermentation (see, *e.g.*, Haft et al, 2014; Sandberg *et al.*, 2014; Lennen and Herrgård, 2014; Tenaillon *et al.*, 2012; Minty *et al.*, 2011; Dragosits *et al.*, 2013a,b; Winkler *et al.*, 2014; Wu *et al.*, 2014; LaCroix *et al.*, 2015; Jensen *et al.*, 2015 and 2016; Doukyu *et al.*, 2012; Shenhar *et al.*, 2012; and Rath and Jawali, 2006).

In addition, Byrne et al., 2012, describes computational modelling of microorganisms such as *E. coli*, proposing combinations of medium compositions and gene-deletion strains for six industrially important byproducts, *e.g.*, succinate. WO 01/05959 (Ajinomoto KK) relates to production of a target substance such as glutamic acid in, *e.g.*, *E. coli* strains. Finally, WO 2016/162442 (Metabolic Explorer) relates to a recombinant microorganism capable of producing 2,4-dihydroxybutyrate, which is characterized by an increased cellular export, and preferably by a decreased cellular import, of 2,4-dihydroxybutyrate.

Despite these and other advances in the art, there is still a need for bacterial cells with improved tolerance to chemicals of interest for bio-based production, such as aliphatic diacids and other compounds. It is an object of the invention to provide such bacterial cells.

SUMMARY OF THE INVENTION

It has been found by the present inventors that certain genetic modifications unexpectedly improve the tolerance of bacterial cells, such as those of, *e.g.*, the *Escherichia* genera, to certain chemical compounds, particularly aliphatic diacids (herein also referred to as "aliphatic dicarboxylic acids").

Accordingly, the invention provides bacterial cells with improved tolerance to at least one aliphatic diacid, as well as bacterial cells which are capable of producing an aliphatic diacid and have improved tolerance to the aliphatic diacid. Particularly contemplated are glutaric acid, adipic acid, succinic acid, muconic acid, fumaric acid, itaconic acid, malic acid, malonic acid, maleic acid, glucaric acid, pimelic acid, suberic acid, sebacic acid, 2,5-furandicarboxylic acid, terephthalic acid, azelaic acid, mesaconic acid, citraconic acid, tartaric acid, tartronic acid, diaminopimelic acid, and glutaconic acid.

The invention also relates to compositions comprising such bacterial cells and one or more aliphatic diacids, methods of preparing or screening for such bacterial cells, and methods of producing aliphatic diacids using such bacterial cells.

These and other aspects and embodiments are described in more detail below.

DETAILED DISCLOSURE OF THE INVENTION

In the present work, glutaric acid and adipic acid were selected for performing adaptive laboratory evolutions. Based on the findings reported herein, various aspects of the invention provide for genetically modified bacterial host cells with a higher tolerance to one or more diacids or other compounds. When transformed with a recombinant biosynthetic pathway for producing the diacid from a carbon source, the genetically modified bacterial host cells of the invention result in improved production of the diacid from carbon feedstock, since they maintain robust metabolic activity in the presence of higher concentrations of the diacid than the parent cells. For example, it was found that a reduced expression of *kgtP* improved tolerance to glutaric acid, implicating KgtP, an α-ketoglutarate importer, as being a direct importer for glutarate.

So, in a first aspect, a bacterial cell is provided, comprising a biosynthetic pathway for producing an aliphatic dicarboxylic acid and at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*, or a combination of any thereof. In one preferred embodiment, the genetic modification reduces the expression of *kgtP*. In another preferred embodiment, the at least one genetic modification reduces the expression of *ybjL, proV, proW, proX, sspA* or a combination of any thereof. For example, the bacterial cell may comprise genetic modifications which reduce the expression of *kgtP, proV* and *ybjL; kgtP* and *proV; kgtP* and *ybjL;* or *kgtP* and *sspA*. Non-limiting examples of genetic modifications include a knock-down or knock-out of the endogenous gene. In a particular embodiment, the genetic modification is a knock-out. The genetic modification may, for example, provide for an increased growth rate, a reduced lag time, or both, of the cell in the presence of at least one of glutaric acid and adipic acid as compared to a control, *e.g.*, the bacterial cell without the genetic modification.

In a second aspect, a bacterial cell is provided, genetically modified from a parent bacterial cell so as to comprise one or more of

(a) a mutant SpoT, comprising at least one mutation in the threonyl-tRNA synthetase GTPase and SpoT (TGS) domain corresponding to amino acid residues I388 to T447 and/or the linker segment between the TGS and the aspartokinase, chorismate mutase and TyrA (ACT) domain corresponding to amino acid residues A448 to T621, optionally in one or more amino acid residues selected from A451, R236, V422, W457, N454, D580, M247, T442, S434, N601, I602 and R603;

(b) a mutant PolB, comprising a mutation in amino acid residue R477;

(c) a mutant RpoC, comprising a mutation in at least one of the amino acid residues corresponding to H419 and P64;

(d) a mutant RpoB, comprising a mutation in an amino acid residue corresponding to K203;

(e) a mutant Rnt, comprising a mutation in at least one of the amino acid residues corresponding to Q179, A27, F194 and A180;

(f) a mutant SapC, comprising a mutation in the amino acid residue corresponding to G79; and

(g) increased expression of PyrE as compared to the parent bacterial cell;

optionally in combination with a knock-down or knock-out of (i) at least one endogenous gene selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*; or (ii) a combination of two or more endogenous genes selected from *kgtP, proV, ybjL* and *sspA*, such as *kgtP, proV* and *ybjL*; *kgtP* and *proV*; *kgtP* and *ybjL*; and *kgtP* and *sspA*,

wherein the genetic modification provides for an increased growth rate, a reduced lag time, or both, in the presence of at least one of glutaric acid and adipic acid as compared to the parent bacterial cell.

The bacterial cell, may, for example, comprise (a) at least one mutant protein selected from the group consisting of SpoT-V422A, SpoT-A451D, SpoT-A451V, SpoT-W457C, SpoT-N454H, SpoT-D580Y, SpoT-R236L, SpoT-R236S, SpoT-M247K, SpoT-NIR(601-603)S, SpoT-T442I, SpoT-S434L, PolB-R477G, RpoC-H419P, RpoC-P64L, RpoB-K203T, Rnt-Q179P, Rnt-A27T, Rnt-F194L, Rnt-A180T, SapC-G79W; and/or (b) a mutation in *rph* or the *pyrE/rph* intergenic region which increases the expression of PyrE.

The bacterial cell of any aspect or embodiment may further comprise a recombinant biosynthetic pathway for producing at least one of glutaric acid, adipic acid, succinic acid, muconic acid, fumaric acid, itaconic acid, malic acid, malonic acid, maleic acid, glucaric acid, pimelic acid, suberic acid, sebacic acid, 2,5-furandicarboxylic acid, terephthalic acid, or azelaic acid, mesaconic acid, citraconic acid, tartaric acid, tartronic acid, diaminopimelic acid and glutaconic acid.

Also provided is a process for preparing a recombinant bacterial cell for producing an aliphatic dicarboxylic acid, the process comprising genetically modifying an *E. coli* cell to (a) introduce a recombinant biosynthetic pathway for producing the aliphatic dicarboxylic acid, and (b) knock-down or knock-out at least one endogenous gene selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*; such as a combination of two or more endogenous genes selected from *kgtP, proV, ybjL* and *sspA*; such as a combination selected from *kgtP, proV* and *ybjL*; *kgtP* and *proV*; *kgtP* and *ybjL* and *kgtP* and *sspA*, and/or (c) express a mutant of at least one of SpoT, PolB, RpoC, RpoB, Rnt and SapC and/or increase the expression of PyrE; wherein steps (a), (b) and (c) can be performed in any order.

Also provided is a process for improving the tolerance of a bacterial cell to an aliphatic dicarboxylic acid comprising genetically modifying the bacterial cell to (a) knock-down or knock-out at least one endogenous gene selected from the group consisting of kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG, and yeaR; such as a combination of two or more endogenous genes selected from kgtP, proV, ybjL and sspA; such as a combination selected from kgtP, proV and ybjL; kgtP and proV; kgtP and ybjL and kgtP and sspA; and/or (b) express a mutant of at least one of SpoT, PolB, RpoC, RpoB, Rnt and SapC and/or increase the expression of PyrE, wherein steps (a) and (b) are performed in any order.

The bacterial cell may, for example, be derived from the *Escherichia, Lactobacillus, Lactococcus, Bacillus, Pseudomonas, Corynebacterium*, *Deinococcus* or *Ralstonia* species, such as the *Escherichia coli* species.

Also provided is a method for producing an aliphatic dicarboxylic acid, comprising culturing such genetically modified bacterial cells in the presence of a carbon source, and, optionally, isolating the aliphatic dicarboxylic acid.

Also provided is a composition comprising glutaric acid or adipic acid at a concentration of at least 5 g/L and a plurality of bacterial cells of the *Escherichia genus* genetically modified to (a) knock-down or knock-out at least one endogenous gene selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*; such as a combination of two or more endogenous genes selected from *kgtP, proV, ybjL* and *sspA*; such as a combination selected from *kgtP, proV* and *ybjL*; *kgtP* and *proV*; *kgtP* and *ybjL* and *kgtP* and *sspA*; and/or (b) express a mutant of at least one of SpoT, PolB, RpoC, RpoB, Rnt and SapC and/or increase expression of PyrE.

In embodiments where the bacterial cell comprises a biosynthetic pathway for producing an aliphatic dicarboxylic acid, the pathway may, for example, comprise

(a) a lysine monooxygenase, a 5-aminovaleramidase, a 5-aminovalerate transaminase, and a glutaraldehyde semialdehyde dehydrogenase;

(b) a reversible 3-oxoadipyl-CoA thiolase, a 3-hydroxyacyl-CoA dehydrogenase, an enoyl-CoA hydratase, an enoyl-CoA reductase, and either a terminal carboxyacyl-CoA thioesterase, or a terminal carboxyacyl-CoA phosphotransferase and a reversible alkyl-1,n-dicarboxylate kinase, where n is the carbon chain length of the product; and, optionally, a malonyl-CoA or glutaryl-CoA transferase; or

(c) a 2-dehydro-3-deoxy-D-arabinoheptonate-7-phosphate synthase, a 3-dehydroquinate synthase, a 3-dehydroxyquinate dehydratase, a dehydroshikimic acid dehydratase, a protocatechuate decarboxylase, and a catechol 1,2-dioxygenase.

Definitions

Unless otherwise indicated or contradicted by context, a "diacid" as used herein is an aliphatic dicarboxylic acid of the general formula COOH-R-COOH (I), where R is an alkyl chain. An "aliphatic diacid" or "aliphatic dicarboxylic acid" herein refers to an organic compound comprising an aliphatic carbon chain to which two or more carboxyl (-COOH) groups are attached, and includes linear aliphatic diacids, as well as derivatives thereof. Aliphatic diacids suitable for production in bacteria typically comprise from 3 to 12 carbon atoms, preferably 3 to 10 carbon atoms, more preferably 3 to 8 carbon atoms, even more preferably, 4 to 7 or 5 to 8 carbon atoms, and, most preferably, 5 to 7 carbon atoms, and optionally comprises one or more heteroatoms or other substituents. Examples of heteroatoms include oxygen (*e.g.*, in the form of an oxo group, a.k.a. keto group), nitrogen, sulphur and halogens. Examples of other substituents include hydroxyl groups, amino groups, carboxyl groups, and alkyl groups. Preferred aliphatic diacids include, but are not limited to, glutaric acid, adipic acid, succinic acid, muconic acid, fumaric acid, itaconic acid, malic acid, malonic acid, maleic acid, glucaric acid, pimelic acid, suberic acid, sebacic acid, 2,5-furandicarboxylic acid, terephthalic acid, or azelaic acid, mesaconic acid, citraconic acid, tartaric acid, tartronic acid, diaminopimelic acid and glutaconic acid. In some embodiments, the aliphatic diacid does not comprise any heteroatom substituents. In some embodiments, the aliphatic diacid does not comprise any substituents. Glutaric, adipic, pimelic and sebacic acid are particularly preferred.

As used herein, a "recombinant biosynthetic pathway" for a compound of interest refers to an enzymatic pathway resulting in the production of a compound of interest in a host cell, wherein at least one of the enzymes is expressed from a transgene, *i.e.*, a gene added to the host cell genome by transformation. In some cases, the recombinant biosynthetic pathway also comprises a deletion of one or more native genes in the host cell. The compound of interest is typically a diacid, and may be the actual end product or a precursor or intermediate in the production of another end product.

The terms "tolerant" or "improved tolerance", when used to describe a genetically modified bacterial cell of the invention or a strain derived therefrom, refers to a genetically modified bacterial cell or strain that shows a reduced lag time, an improved growth rate, or both, in the presence of a diacid than the parent bacterial cell or strain from which it is derived, typically at concentrations of 1 g/L, such as at least 2 g/L or higher, such as at least 5 g/L or higher, such as at least 10 g/L or higher, such as at least 20 g/L or higher, such as at least 40 g/L or higher, such as at least 75 g/L or higher, such as at least 100 g/L or higher. An improved growth rate is at least 5%, such as at least 10%, such as at least 20%, such as at least 50%, such as at least 75% higher than that of a control, typically the parent cell or strain. A reduced lag time is at least 10%, such as at least 20%, such as at least 50%, such as at least 75%, such as at least 90% shorter than that of a control, typically the parent cell or strain.

The term "gene" refers to a nucleic acid sequence that encodes a cellular function, such as a protein, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. An "endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "transgene" is a gene, native or heterologous, that has been introduced into the genome by a transformation procedure. Genes names are herein set forth in italicised text with a lower-case first letter (*e.g.*, *metJ*) whereas protein names are set forth in normal text with a capital first letter (*e.g.*, MetJ).

As used herein the term "coding sequence" refers to a DNA sequence that encodes a specific amino acid sequence.

The term "native", when used to characterize a gene or a protein herein with respect to a host cell, refers to a gene or protein having the nucleic acid or amino acid sequence as found in the host cell.

The term "heterologous", when used to characterize a gene or protein with respect to a host cell, refers to a gene or protein which has a nucleic acid or amino acid sequence not normally found in the host cell.

As used herein the term "transformation" refers to the transfer of a nucleic acid fragment, such as a gene, into a host cell. Host cells containing a gene introduced by transformation or a "transgene" are referred to as "transgenic" or "recombinant" or "transformed" cells.

As used herein, a "genetic modification" or "genetically modified" refers to the introduction a genetically inherited change in the host cell genome. Examples of changes include mutations in genes and regulatory sequences, coding and non-coding DNA sequences. "Mutations" include deletions, substitutions and insertions of one or more nucleotides or nucleic acid sequences in the genome. Other genetic modifications include the introduction of heterologous genes or coding DNA sequences on a plasmid and/or into a chromosome by recombinant techniques. In one embodiment, the genetic modification is in a chromosome.

The term "expression", as used herein, refers to the process in which a gene is transcribed into mRNA, and may optionally include the subsequent translation of the mRNA into an amino acid sequence, *i.e.*, a protein or polypeptide.

As used herein, "reduced expression" or "downregulation" of an endogenous gene in a host cell means that the levels of the mRNA, protein and/or protein activity encoded by the gene are significantly reduced in the host cell, typically by at least 25%, such as at least 50%, such as at least 75%, such as at least 90%, such as at least 95%, as compared to a control. Typically, when the reduced expression is obtained by a genetic modification in the host cell, the control is the unmodified host cell. Sometimes, *e.g.*, in the case of gene knock-out, the reduction of native mRNA and functional protein encoded by the gene is higher, such as 99% or greater.

"Increased expression", "upregulation", "overexpressing" or the like, when used in the context of a protein or activity described herein, means increasing the protein level or activity within a bacterial cell. An up-regulation of an activity can occur through, *e.g.*, increased activity of a protein, increased potency of a protein or increased expression of a protein. The protein with increased activity, potency or expression can be encoded by genes disclosed herein.

Genetic modifications resulting in a reduced expression of a target gene/protein can include, *e.g.*, knock-down of the gene (*e.g.*, a mutation in a promoter or other expression control sequence that results in decreased gene expression), a knock-out or disruption of the gene

(*e.g.*, a mutation or deletion of the gene that results in 99 percent or greater decrease in gene expression), a mutation or deletion in the coding sequence which results in the expression of non-functional protein, and/or the introduction of a nucleic acid sequence that reduces the expression of the target gene, *e.g.* a repressor that inhibits expression of the target or inhibitory nucleic acids (*e.g.* CRISPR/dCas9, antisense RNA, etc.) that reduces the expression of the target gene.

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 4th ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 2012; and by Silhavy, T. J., Bennan, M. L. and Enquist, L. W. Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1984; and by Ausubel, F. M. *et al.*, In Current Protocols in Molecular Biology, published by John Wiley & Sons (1995); and by Datsenko and Wanner, 2000; and by Baba *et al.*, 2006; and by Thomason *et al.*, 2007.

A "conservative" amino acid substitution in a protein is one that does not negatively influence protein activity. Typically, a conservative substitution can be made within groups of amino acids sharing physicochemical properties, such as, *e.g.*, basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagines), hydrophobic amino acids (leucine, isoleucine, valine and methionine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, and threonine). Most commonly, substitutions can be made between Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly. Other preferred substitutions are set out in Table 1 below.

Table 1. *Examples of amino acid substitutions.*

Original amino acid	Examples of substitutions	Preferred substitution
Ala (A)	val; leu; ile	Val
Arg (R)	lys; gln; asn	Lys
Asn (N)	gln; his; asp, lys; arg	Gln
Asp (D)	glu; asn	Glu
Cys (C)	ser; ala	Ser
Gln (Q)	asn; glu	Asn

Glu (E)	asp; gln	Asp
Gly (G)	Ala	Ala
His (H)	asn; gln; lys; arg	Arg
Ile (I)	leu; val; met; ala; phe; norleucine	Leu
Leu (L)	norleucine; ile ; val; met; ala; phe	Ile
Lys (K)	arg; gln; asn	Arg
Met (M)	leu; phe; ile	Leu
Phe (F)	leu; val; ile; ala; tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	thr	Thr
Thr (T)	Ser	Ser
Trp (W)	tyr; phe	Tyr
Tyr (Y)	trp; phe ; thr; ser	Phe
Val (V)	ile; leu; met; phe; ala; norleucine	Leu

Specific embodiments of the invention

As described herein, the invention provides bacterial cells with improved tolerance to one or more diacids, as well as related processes and materials for producing and using such bacterial cells.

1) Genetic modifications

The genetic modifications according to the invention include those resulting in reduced expression of genes, *e.g.*, by gene knock-down or knock-out, herein referred to as "Group 1 modifications"; as well as silent mutations in coding or non-coding regions and non-silent (*i.e.*, coding) mutations in coding regions, herein referred to as "Group 2 modifications"; and combinations thereof.

In a preferred embodiment, the one or more genetic modifications provide for an increased growth rate, a reduced lag time, or both, of the bacterial cell in the presence of at least one of glutaric and adipic acid as compared to the bacterial cell without the genetic modification, *e.g.*, the parent or wild-type bacterial cell. The glutaric and/or adipic acid may be present in the growth medium at, *e.g.*, a concentration of at least about 1 g/L, such as at least about 2

g/L, such as at least about 5 g/L, such as at least about 10 g/L, such as at least about 20 g/L.

a) Group 1 modifications

In one aspect, the bacterial cell has at least one genetic modification which reduces expression an endogenous gene selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*.

For example, in one embodiment, the expression of one or more of *kgtP, ybjL, proV, proW, proX, proQ* and *sspA,* such as *kgtP, ybjL, proV* or *sspA,* is reduced. In one specific embodiment, the expression of *kgtP* is reduced, optionally wherein the expression of *lysP* is not reduced. In one specific embodiment, the expression of *ybjL* is reduced. In one specific embodiment, the expression of *sspA* is reduced. In one specific embodiment, the expression of *proV, proW, proX* or *proQ,* such as *e.g. proV,* is reduced, optionally wherein the expression of *marR* is not reduced. In another specific embodiment, the expression of *cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, or *yeaR* is reduced.

In another aspect, there is provided a bacterial cell which comprises genetic modifications reducing the expression of two or more endogenous genes, wherein at least one gene is selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR.* In one embodiment, the bacterial cell comprises a genetic modification reducing the expression of *kgtP* but no genetic modification which reduces the expression of *lysP*. In one embodiment, the bacterial cell comprises a genetic modification reducing the expression of *proV* but no genetic modification which reduces the expression of *marR*. In one embodiment, the bacterial cell comprises genetic modifications which reduce the expression of at least two genes selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR.*

In one embodiment, the genetic modifications reduce the expression of *kgtP* and one or more other endogenous genes, optionally selected from *ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG* and *yeaR*, such as from *ybjL, proV, proW, proX,* and *sspA*, optionally wherein the other endogenous genes do not comprise *lysP.* In separate and specific embodiments, the bacterial cell comprises genetic modifications which reduce the expression of *kgtP* and *ybjL*, *kgtP* and *proV, proV* and *ybjL*, or *kgtP* and *sspA*.

In one embodiment, the genetic modifications reduce the expression of *kgtP* and two or more endogenous genes selected from *ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG* and *yeaR*, wherein at least one endogenous gene is selected from *ybjL, proV, proW, proX,* and *sspA*. In one specific embodiment, the bacterial cell comprises genetic modifications which reduce the expression of *kgtP*, *proV* and *ybjL*.

In other separate and specific embodiments, the bacterial cell comprises:

- a first genetic modification which reduces the expression of *ybjL,* and a second genetic modification which reduces the expression of a gene selected from *kgtP, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*;
- a first genetic modification which reduces the expression of *proV* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*;
- a first genetic modification which reduces the expression of *proW* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *proX* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *proQ* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *cspE* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*;
- a first genetic modification which reduces the expression of *rfaE* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*;

- a first genetic modification which reduces the expression of *yfbP* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *yfjM* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*;
- a first genetic modification which reduces the expression of *pstS* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *pstA* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *pstB* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *pstC* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *rph* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *rpoS* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, sspA, tdk, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *sspA* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, tdk, uvrB, ycjG*, and *yeaR;*

- a first genetic modification which reduces the expression of *tdk* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, uvrB, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *uvrB* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, ycjG*, and *yeaR;*
- a first genetic modification which reduces the expression of *ycjG* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB*, and *yeaR; or*
- a first genetic modification which reduces the expression of *yeaR* and a second genetic modification which reduces the expression of a gene selected from of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB*, and *ycjG.*

In one specific embodiment, either one or both of the first and second genetic modifications is a knock-out of the gene, optionally a deletion. In an alternative embodiment at least one of the first and second genetic modifications is a knock-down of the gene.

In one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein the genetic modification is a knock-down of the one or more endogenous genes, resulting in at least 25%, such as at least 50%, such as at least 75%, such as at least 90%, such as at least 95%, reduction in the level of mRNA encoded by the gene.

In one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein the genetic modification is a knock-down of the one or more endogenous genes, resulting in at least 25%, such as at least 50%, such as at least 75%, such as at least 90%, such as at least 95%, reduction in the level of protein encoded by the gene.

In one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein the genetic modification is a knock-out of the one or more endogenous genes.

Knock-down or knock-out of a gene can be accomplished by any method known in the art for bacterial cells, and include, *e.g.*, lambda Red mediated recombination, P1 phage transduction, and single-stranded oligonucleotide recombineering/MAGE technologies (see, *e.g.*, Datsenko and Wanner, 2000; Thomason *et al.*, 2007a,b; Wang *et al.*, 2009). Typically, a knock-down of a gene can be accomplished by, for example, a mutation in the promoter region resulting in decreased transcription, a deletion or mutation in the coding region of the gene resulting in a reduced or fully or substantially eliminated activity of the protein, or by the presence of antisense sequences that interfere with transcription or translation of the gene, resulting in reduced expression of the protein. Preferably, the knocking-down of a gene results in at least 20% reduction in the expression level of the gene product in the bacterial cell, such as at least 30%, such as at least 40%, such as at least 50%, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95% or higher.

A knock-out of a gene includes elimination of a gene's expression, such as by introducing a mutation in the coding sequence and/or promoter so that at least a portion (up to and including all) of the coding sequence and/or promoter is disrupted, shifted or deleted, resulting in loss of expression of the protein, or expression only of a non-functional mutant or non-functional fragment of the endogenous protein. As used herein, the symbol "Δ", *i.e.*, the greek uppercase letter for "delta", denotes a deletion of an endogenous gene. Preferably, a knock-out of a gene results in 1% or less of the native gene product being detectable, such as no detectable gene product.

b) Group 2 modifications

In certain embodiments, a mutant protein is expressed in the bacterial cell, *e.g.*, from a mutated version of an endogenous gene, or from a transgene encoding the mutant protein.

In one aspect, the bacterial cell comprises a Group 2 modification, *e.g.*, a mutation in one or more of SpoT, PolB, RpoC, RpoB, Rnt and SapC; an increased expression of one or more of SpoT, PolB, RpoC, RpoB, Rnt and SapC; and/or a mutation in *rph* or the *pyrE/rph* intergenic region which increases the expression of *pyrE,* wherein the one or more mutations improve tolerance to at least one aliphatic diacid such as, *e.g.* glutaric or adipic acid. Preferably, the bacterial cell further comprises a Group 1 modification according to any aspect or embodiment herein.

In one embodiment, the Group 2 modification comprises a mutant SpoT, comprising one or more mutations. The mutations may be located, for example, in the threonyl-tRNA synthetase GTPase and SpoT (TGS) domain corresponding to amino acid residues I388 to

T447, or the linker region between the TGS domain and the aspartokinase, chorismate mutase and TyrA (ACT) domain corresponding to A448 to T621 in *E. coli* SpoT. Without being limited to theory, since the mutations identified are near the TGS domain which is involved in nucleotide binding, at least some of the mutations may decrease the ppGpp synthetase activity of SpoT, *e.g.*, by decreasing its binding affinity to substrates such as ATP or (p)ppGpp, or increasing its binding affinity to products such as GTP, AMP, or GDP. This may,in turn, reduce sensitivity of the cells to accumulating ppGpp and delay the onset of the stringent response. The stringent response may be activated under general stress conditions such as in high concentrations of diacids and might prevent growth under such conditions. In one embodiment, the SpoT mutant comprises a mutation in one or more amino acid residues selected from those corresponding to R236, M247, V422, S434, T442, A451, N454, W457, D580, N601, I602 and R603 in *E. coli* SpoT. In one embodiment, the mutant SpoT comprises at least one amino acid substitution selected from V422A, A451D, A451V, W457C, N454H, D580Y, R236L, R236S, M247K, NIR(601-603)S, T442I, and S434L or a conservative substitution of any thereof. In a specific embodiment, the mutant SpoT comprises a mutation in A451 or R236, *e.g.*, an amino acid substitution selected from A451D, A451V, R236L and R236S, or a conservative substitution thereof, *e.g.*, selected from A451E, A451N, A451G, A451A, A451L, A451I, R236I, R236V, R236T, R236A, R236N and R236G.

In one embodiment, the Group 2 modification comprises a mutant PolB comprising one or more mutations. The mutation may be located, *e.g.*, in the residue corresponding to R477 in *E. coli* PolB, and may be an amino acid substitution such as R477G or a conservative substitution thereof, *e.g.*, R477A, R477D or R477S.

In one embodiment, the Group 2 modification comprises a mutant RpoC comprising one or more mutations. The mutation may be located in, *e.g.*, the residue corresponding to H419 and/or P64 in *E. coli* RpoC, and may be an amino acid substitution such as H419P, P64L, or a conservative substitution thereof, *e.g.*, H419A, P64I, P64V, P64M, P64A or P64F. Without being limited to theory, since some of these residues (*e.g.*, H419) are close to residues involved in ppGpp-binding, at least some of them may decrease ppGpp binding to RpoC which in turn may reduce sensitivity of the cells to accumulating ppGpp and delay the onset of the stringent response.

In one embodiment, the Group 2 modification comprises a mutant RpoB comprising one or more mutations. The mutation may be located in, *e.g.*, the residue corresponding to K203 in *E. coli* RpoB, and may be an amino acid substitution such as K203T or a conservative substitution thereof, *e.g.*, K203S or K203A. Without being limited to theory, since residue K203 is near the entrance for dsDNA, it may interact with phosphate on dsDNA, possibly reducing premature transcription termination.

In one embodiment, the Group 2 modification comprises a mutant Rnt comprising one or more mutations. The mutations may be located, for example, in or close to catalytic residues of conserved exonuclease motifs corresponding to positions 23, 25, 181, and 186 in *E. coli* Rnt. In one embodiment, the Rnt mutant comprises a mutation in one or more amino acid residues selected from those corresponding to Q179, A27, F194 and A180. In one embodiment, the mutant Rnt comprises at least one amino acid substitution selected from Q179P, A27T, F194L and A180T or a conservative substitution of any thereof, *e.g.*, Q179A, A27S, A27G, F195I, F195V, F195T, F195A, A180S and A180G.

In one embodiment, the Group 2 modification comprises a mutant SapC comprising one or more mutations. The mutation may be located in, *e.g.*, the residue corresponding to G79 in *E. coli* SapC, and may be an amino acid substitution such as G79W or a conservative substitution thereof, *e.g.*, G79Y or G79F.

In one embodiment, the bacterial cell comprises one or more mutations which increase(s) the expression level or activity of PyrE, optionally in combination with a Group 1 modification. *E. coli* K-12 MG1655 and W3110, plus their common ancestor strain W1485, are known to exhibit pyrimidine starvation in minimal media due to the presence a frameshift mutation occurring in *rph* relative to other *E. coli* strains (Jensen *et al.*, 1993). This mutation disrupts the transcriptional/translational coupling required for efficient translation of *pyrE*, encoding orotate phosphoribosyltransferase in the pyrimidine biosynthesis pathway. Compensatory mutations that correct this deficiency are well-known in the art. One of these mutations is an 82 bp deletion near the 3′ terminus of *rph*, due to presence of two homologous GCAGAAGGC sequences flanking this 82 bp region (Conrad *et al.*, 2009). In addition to the 82 bp deletion, a 1 bp deletion at coordinate 3815809 in the pyrE/rph intergenic region has previously been encountered in strains evolved for growth on a minimal glucose medium (LaCroix *et al.*, 2015), and a wide array of other frameshift mutations, substitutions, and coding mutations near the 3′ terminus of *rph* were encountered in a short-term selection/evolution of combinatorial mutant libraries in minimal medium at an elevated temperature of 42°C (Sandberg *et al.*, 2014). Without being limited to theory, all of these mutations can serve the same function of increasing expression of PyrE, with the selective pressure for these mutations being even stronger in minimal media with particular imposed stresses (certain chemicals or heat) than in minimal media alone. In one embodiment, the bacterial cell comprises mutations in *rph* or the *pyrE*/*rph* intergenic region, such as, *e.g.*, the 82 bp deletion near the 3′ terminus of *rph*, the 1 bp deletion in the intergenic region between *pyrE* and *rph*, or both. In one embodiment, increased expression of PyrE is achieved by transforming the bacterial cell with a transgene expressing the endogenous protein. Increased expression may be obtained by causing an up-regulation through increased expression of a protein, the copy number of a gene or genes encoding the protein may be

increased. Alternatively, a strong and/or inducible promoter can be used to direct the expression of the gene, the gene being expressed either as a transient expression vehicle or homologously or heterologously incorporated into the bacterial genome. In another embodiment, the promoter, regulatory region and/or the ribosome binding site upstream of the gene can be altered to achieve the over-expression. The expression can also be enhanced by increasing the relative half-life of the messenger or other forms of RNA. Any one or a combination of these approaches can be used to effect upregulation of a desired target protein as needed.

In a specific embodiment, the bacterial cell comprises at least one Group 1 modification and at least one Group 2 modification. Non-limiting examples of Group 1 modifications for combination with any one or more of the overexpressed or mutant SpoT, PolB, RpoC, RpoB, Rnt, SapC and *pyrE/rph* include knock-down or knock-out of

i) at least one endogenous gene selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*; and

ii) a combination of two or more endogenous genes selected from *kgtP, proV, ybjL* and *sspA,* such as *kgtP, proV* and *ybjL*; *kgtP* and *proV; kgtP* and *ybjL;* and *kgtP* and *sspA.*

In separate and specific embodiments, the bacterial cell comprises:

- a mutation selected from SpoT-V422A, SpoT-A451D, SpoT-A451V, SpoT-W457C, SpoT-N454H, SpoT-D580Y, SpoT-R236L, SpoT-R236S, SpoT-M247K, SpoT-NIR(601-603)S, SpoT-T442I, SpoT-S434L, PolB-R477G, RpoC-H419P, RpoC-P64L, RpoB-K203T, Rnt-Q179P, Rnt-A27T, Rnt-F194L, Rnt-A180T, and SapC-G79W
- a mutation selected from SpoT-V422A, SpoT-A451D, SpoT-A451V, SpoT-W457C, SpoT-N454H, SpoT-D580Y, SpoT-R236L, SpoT-R236S, SpoT-M247K, SpoT-NIR(601-603)S, SpoT-T442I, SpoT-S434L, PolB-R477G, RpoC-H419P, RpoC-P64L, RpoB-K203T, Rnt-Q179P, Rnt-A27T, Rnt-F194L, Rnt-A180T, and SapC-G79W, and a knock-out or knockdown of at least one of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG,* or *yeaR*, such as *kgtP*.
- a mutation selected from SpoT-V422A, SpoT-A451D, SpoT-A451V, SpoT-W457C, SpoT-N454H, SpoT-D580Y, SpoT-R236L, SpoT-R236S, SpoT-M247K, SpoT-NIR(601-603)S, SpoT-T442I, SpoT-S434L, PolB-R477G, RpoC-H419P, RpoC-P64L, RpoB-K203T, Rnt-Q179P, Rnt-

A27T, Rnt-F194L, Rnt-A180T, and SapC-G79W, and a knock-out or knockdown of at least two of *kgtP, ybjL,* and *proV* (or genes encoding other subunits in the same protein complex, which are *proX* and *proW*), such as *kgtP* and *proV*.

- a mutation selected from SpoT-V422A, SpoT-A451D, SpoT-A451V, SpoT-W457C, SpoT-N454H, SpoT-D580Y, SpoT-R236L, SpoT-R236S, SpoT-M247K, SpoT-NIR(601-603)S, SpoT-T442I, SpoT-S434L, PolB-R477G, RpoC-H419P, RpoC-P64L, RpoB-K203T, Rnt-Q179P, Rnt-A27T, Rnt-F194L, Rnt-A180T, and SapC-G79W, and a knock-out or knockdown of *kgtP, ybjL,* and *proV* (or genes encoding other subunits in the same protein complex, which are *proX* and *proW*) in combination, such as *kgtP, proV,* and *ybjL*.
- a mutation increasing the expression of *pyrE* and a knock-out or knockdown of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG,* or *yeaR*, such as *kgtP*.
- a mutation increasing the expression of *pyrE* and a knock-out or knockdown of at least two of *kgtP, ybjL,* and *proV* (or genes encoding other subunits in the same protein complex, which are *proX* and *proW*), such as *kgtP* and *proV*.
- a mutation increasing the expression of *pyrE* and a knock-out or knockdown of *kgtP, ybjL,* and *proV* (or genes encoding other subunits in the same protein complex, which are *proX* and *proW*) in combination, such as *kgtP*, *proV*, and *ybjL*..

In other separate and specific embodiments, the bacterial cell comprises:

- a combination of mutations SpoT-V422A and RpoC-H419P, and a knock-out or knockdown of *kgtP*.
- a mutation SpoT-V422A, and a knock-out or knockdown of *kgtP* and at least one of *proV*, *proW* and *proX*, and a knock-out, knockdown, or reduction of function mutation in *sspA*.
- a mutation SpoT-A451D, and a knock-out or knockdown of *kgtP*.
- a mutation SpoT-A451D, and a knock-out or knockdown of *kgtP* and at least one of *proV, proW* and *proX*.
- a combination of mutations SpoT-A451D and RpoC-H419P, and a knock-out or knockdown of *kgtP* and at least one of *proV, proW* and *proX*.
- a combination of mutations SpoT-W457C and RpoC-H419P, and a knock-out or knockdown of *kgtP*.
- a mutation SpoT-D580Y, and a knock-out or knockdown of *kgtP* and at least one of *proV, proW* and *proX*.

- a combination of mutations SpoT-R236L and RpoB-K203T, and a knock-out or knockdown of *kgtP* and *sspA*.
- a combination of mutations PolB-R477G and RpoC-P64L, and a knock-out or knockdown of *kgtP* and at least one of *proV, proW* and *proX*.
- a knock-out or knockdown of *kgtP* and *ybjL*, and a mutation that increases the expression of PyrE.
- a knock-out or knockdown of *kgtP* and a mutation that increases the expression of PyrE.
- a knock-out of knockdown of *kgtP*, *ybjL*, and at least one of *nagA* or *nagC*.
- a mutation SpoT-S434L, and a knock-out of knockdown of *kgtP; at least one of proV, proW* and *proX*; *ybjL*; and at least one of *nagC* and *nagA*.
- a combination of mutations SpoT-S434L and ProQ-R80C, and a knock-out of knockdown of *kgtP;* at least one of *proV, proW* and *proX*; *ybjL*,and at least one of *nagC* and *nagA*.
- a mutation SpoT-S434L, and a knock-out or knockdown of *kgtP; at least one of proV, proW* and *proX*; *ybjL*; and at least one of *nagC* and *nagA*; and *proQ*.
- a knock-out of knockdown of *kgtP* and one of *pstS*, *tdk,* and *rpoS,* or any combination thereof.
- a knock-out of knockdown of *kgtP*; *ybjL*; at least one of *proV, proW* and *proX*; and *sspA*.
- a knock-out of knockdown of *kgtP*, *tdk*, and *pstS*.

2) Production pathways

In some aspects, the bacterial cell comprises a recombinant pathway for producing an aliphatic diacid of interest, optionally providing for a production level of at least about 5 g/L of the aliphatic diacid over a predetermined period of time, e.g., about 200h, about 100h, about 72h, about 48h or about 24h. A recombinant pathway can, for example, be added to introduce the capability to produce the diacid in a bacterial cell which does not have a native pathway to do so, typically by transforming the cell with one or more heterologous enzymes catalyzing the desired reaction(s). Alternatively, in cases where the bacterial cell has a native pathway for production of the diacid of interest, a recombinant pathway can nonetheless be introduced in order to increase the production yield, *e.g.*, by overexpressing one or more native enzymes or transforming the cell with heterologous enzymes. In separate and specific embodiments, the recombinant pathway provides for a production level of at least 5 g/L, such as at least 10 g/L or higher, such as at least 20 g/L or higher, such as at least 40 g/L or higher, such as at least 45 g/L or higher, such as at least 75 g/L or higher, such as at least 100 g/L or higher.

So, in one aspect, there is provided a bacterial cell with improved tolerance to at least one aliphatic diacid according to any aspect or embodiment described herein, wherein the bacterial cell further comprises a recombinant biosynthetic pathway for producing an aliphatic diacid of interest, such as, *e.g.*, glutaric acid, adipic acid, succinic acid, muconic acid, fumaric acid, itaconic acid, malic acid, malonic acid, maleic acid, glucaric acid, pimelic acid, suberic acid, sebacic acid, 2,5-furandicarboxylic acid, terephthalic acid, or azelaic acid, mesaconic acid, citraconic acid, tartaric acid, tartronic acid, diaminopimelic acid or glutaconic acid. In principle, any such recombinant biosynthetic pathway which is known in the art can be introduced into the cell by standard recombinant technologies. Some specific, preferred pathways are, however, exemplified below and in Example 1 – see the section entitled "Biological production of diacids" and references cited therein.

It is to be understood that, when a specific enzyme of these biosynthetic pathways is mentioned by name such as, *e.g.*, "lysine monooxygenase", the enzyme may be any characterized and sequenced enzyme, from any species, that have been reported in the literature so long as it provides the desired activity. In some embodiments, the enzyme is an overexpressed gene which is native to the host cell used. In some embodiments, the enzyme is a functionally active fragment or variant of an enzyme which is heterologous or native to the host cell. Also, in some embodiments, the recombinant biosynthetic pathway comprises a knock-down or a knock-out of one or more genes, typically for the purpose of avoiding competing reactions reducing the yield of the desired aliphatic diacid.

So, in one embodiment, the biosynthetic pathway is for producing glutaric acid from glucose, and comprises genes, optionally overexpressed and/or heterologous, encoding:

- a lysine monooxygenase;
- a 5-aminovaleramidase;
- a 5-aminovalerate transaminase, and
- a glutarate semialdehyde dehydrogenase.

The bacterial cell may further be modified by one or more of

(i) introducing feedback resistance mutations in native genes corresponding to DapA (4-hydroxytetrahydrodipicolinate synthase) and LysC (asparate kinase III), optionally also overexpressing the modified proteins; and

(ii) knockdown or knock-out of native genes corresponding to *cadA* and *ldcC*;

(iii) constitutive overexpression of lysine biosynthesis, *e.g.*, via a dapA promoter replacement; and

(iv) knock-down or knock-out of native genes corresponding to *speE, speG, patA* and *puuPA*.

In one embodiment, the biosynthetic pathway is for producing adipic acid and comprises

- a reversible 3-oxoadipyl-CoA thiolase (*e.g.*, PaaJ from *E. coli*),
- a 3-hydroxyacyl-CoA dehydrogenase (*e.g.*, PaaH1 from *Ralstonia eutropha*),
- an enoyl-CoA hydratase (*e.g.*, h16_AA307 gene product from *Ralstona eutropha* H16),
- a *trans*-enoyl-CoA reductase (*e.g.*, Ter from *Euglena gracilis*),
- a phosphate butyryltransferase (*e.g.*, Ptb from *Clostridium acetobutylicum*), and
- a butyryl kinase (*e.g.*, Buk1 from *Clostridium acetobutylicum*)

The bacterial cell may further be modified to knock-down or knock-out one or more native genes corresponding to *ptsG*, *poxB, pta*, *sdhA*, and *iclR*.

In one embodiment, the biosynthetic pathway is for producing adipic acid and comprises

- a 3-oxoadipyl-CoA thiolase (*e.g.*, PaaJ from *E. coli*),
- a 3-hydroxyacyl-CoA dehydrogenase (*e.g.* PaaH from *E. coli*)
- an enoyl-CoA hydratase (*e.g.* PaaF from *E. coli*)
- an enoyl-CoA reductase (*e.g.* Ter from *Treponema denticola*)
- an acyl-CoA thioesterase (*e.g.* Acot8 from *Mus musculus*)

The bacterial cell may further be modified to knock-down or knock-out one or more native genes corresponding to *pta*, *poxB*, *ldhA*, and *adhE*.

In one embodiment, the biosynthetic pathway is for producing pimelic acid and comprises feeding glutaric acid and

- a glutaryl-CoA transferase (*e.g.*,Cat1 from *Clostridium kluyveri*),
- a 3-oxoadipyl-CoA thiolase (*e.g.*, PaaJ from *E. coli*),
- a 3-hydroxyacyl-CoA dehydrogenase (*e.g.* PaaH from *E. coli*)
- an enoyl-CoA hydratase (*e.g.* PaaF from *E. coli*)
- an enoyl-CoA reductase (*e.g.* Ter from *Treponema denticola*)
- an acyl-CoA thioesterase (*e.g.* Acot8 from *Mus musculus*)

The bacterial cell may further be modified to knock-down or knock-out one or more native genes corresponding to *pta*, *poxB*, *ldhA*, *adhE,* and *frdA*.

In one embodiment, the biosynthetic pathway is for producing sebacic acid and comprises

- a glutaryl-CoA transferase (*e.g.*,Cat1 from *Clostridium kluyveri*),
- a 3-oxoadipyl-CoA thiolase (*e.g.*, DcaF from *Acinetobacter sp.* ADP1),
- a 3-hydroxyacyl-CoA dehydrogenase (*e.g.* DcaH from *Acinetobacter sp.* ADP1)
- an enoyl-CoA hydratase (*e.g.* DcaE from *Acinetobacter sp.* ADP1)
- an enoyl-CoA reductase (*e.g.* Ter from *Treponema denticola*)
- an acyl-CoA thioesterase (*e.g.* Acot8 from *Mus musculus*)

The bacterial cell may further be modified to knock-down or knock-out one or more native genes corresponding to *pta*, *poxB*, *ldhA*, *adhE, frdA*, and native acyl-CoA thioesterases including *yciA*, *ybgC*, *ydiI*, *tesA*, *fadM*, and *tesB*.

In one embodiment, the biosynthetic pathway is for producing adipic acid and comprises

- a 3-oxoadipyl-CoA thiolase/β-ketothioase (*e.g.* from *Thermobifida fusca* B6),
- a 3-hydroxyacyl-CoA dehydrogenase (*e.g.* from *Thermobifida fusca* B6)
- an enoyl-CoA hydratase (*e.g.* from *Thermobifida fusca* B6)
- an enoyl-CoA reductase (*e.g.* from *Thermobifida fusca* B6)
- a succinyl-CoA synthetase (*e.g.* Tfu_2577 and Tfu_2576 from *Thermobifida fusca* B6)

The bacterial cell may further be modified to knock-down or knock-out one or more native genes corresponding to *pta*, *poxB*, *ldhA*, *adhE*, *ptsG*, *sdhA*, and *iclR*.

In one embodiment, the biosynthetic pathway is for producing adipic acid via whole cell bioconversion from supplied medium to long chain free fatty acids (C_{12}-C_{16}) and comprises combinations of

- a heterologously expressed or increased native activity of a 6-oxohexanoic acid dehydrogenase
- a heterologously expressed or increased native activity of an omega exo fatty acid dehydrogenase
- a heterologously expressed or increased native activity of a 6-hydroxyhexanoic acid dehydrogenase
- a heterologously expressed or increased native activity of an omega hydroxyl fatty acid dehydrogenase
- a heterologously expressed or increased native activity of a hexanoate synthase
- a heterologously expressed or increased native activity of a monooxygenase
- a heterologously expressed or increased native activity of a monooxygenase reductase

- a heterologously expressed or increased native activity of a fatty acid oxidase
- a heterologously expressed or increased native activity of an acyl-CoA ligase
- a heterologously expressed or increased native activity of an acyl-CoA oxidase
- a heterologously expressed or increased native activity of an enoyl-CoA reductase
- a heterologously expressed or increased native activity of a 3-L-hydroxyacyl-CoA dehydrogenase
- a heterologously expressed or increased native activity of an acetyl-CoA C-acetyltransferase

In one embodiment, the biosynthetic pathway is for producing muconic acid and comprises

- a native or heterologously expressed pathway comprising a 2-dehydro-3-deoxy-D-arabinoheptonate 7-phosphate (DAHP) synthase, a 3-dehydroquinate synthase, and a 3-dehydroxyquinate dehydratase
- a dehydroshikimic acid dehydratase (*e.g.* PobA from *Pseudomonas putida* KT2440)
- a protocatechuate decarboxylase (*e.g.* AroY from *Klebisella pneumoniae*)
- a catechol 1,2-dioxygenase (*e.g.* CatA from *Actinetobacter sp.* ADP1)

The bacterial cell may further be modified to increase the flux toward precursors for DAHP (erythrose 4-phosphate and phosphoenolpyruvate), such as by knock-down or knock-out of genes corresponding to *E. coli ptsH, ptsI, crr,* and *pykF*; by overexpressing genes corresponding to *ubiC*, *aroF, aroE,* and *aroL* (or feedback-resistant mutants thereof), or combinations thereof.

Some bacteria contain a native pathway for production of a diacid, avoiding the necessity for a recombinant pathway. These include, for example, *Actinobacillus succinogenes* (succinic acid), *Mannheimia succiniproducens* (succinic acid), *Thermobifida fusca* (malic acid), and *Escherichia coli* (fumaric and malic acids), among numerous others.

3) Processes

In one aspect, there is provided a process for preparing a recombinant bacterial cell, *e.g.*, an *E. coli* cell. Also provided is a process for improving the tolerance of a bacterial cell, *e.g.*, an *E. coli* cell, to a diacid. Also provided is a method of identifying a bacterial cell which is tolerant to at least one diacid. Also provided is a process for preparing a recombinant bacterial cell, *e.g.*, an *E. coli* cell, for producing a diacid.

These processes may comprise one or more steps of genetically modifying a bacterial cell to knock-down or knock-out one or more endogenous genes of any aspect or embodiment of the Group 1 modifications and/or introducing one or more mutations in the endogenous

protein(s) or gene(s) of any Group 2 aspect or embodiment. This can be achieved by, *e.g.*, transforming the bacterial cell with genetic constructs, *e.g.*, vectors, antisense nucleic acids or siRNA, which result, *e.g.*, in the knock-out or knock-down of a gene, introduce a mutation into an endogenous gene, or which encode the mutated protein from a transgene.

The genetic constructs, particularly vectors, can also comprise suitable regulatory sequences, typically nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters (*e.g.*, constitutive promoters or inducible promoters), translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

Alternatively, bacterial cells can be exposed to selection pressure (as described in the Examples) or to conditions which introduce random mutations in endogenous genes, and bacterial cells which comprise one or more Group 1 and/or Group 2 modifications according to any preceding aspects and embodiments can then be identified. Typically, this involves preparing a population of the genetically modified bacterial cell, having different Group 1 and/or Group 2 modifications, and then selecting from this population any bacterial cell which has an improved tolerance to a diacid at a predetermined concentration.

In one specific embodiment, the Group 1 modification is a knock-down or knock-out of one or more endogenous genes selected from *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR* or, *e.g.*, a knock-down or knock-out of *kgtP* in combination one or more other genes, *e.g.*, *ybjL, proV* and/or *sspA*. In one specific embodiment, the Group 2 modification is a mutation in at least one endogenous protein or gene selected from SpoT, PolB, RpoC, RpoB, Rnt or SapC, such as *e.g.*, at least one mutant protein selected from the group consisting of SpoT-V422A, SpoT-A451D, SpoT-A451V, SpoT-W457C, SpoT-N454H, SpoT-D580Y, SpoT-R236L, SpoT-R236S, SpoT-M247K, SpoT-NIR(601-603)S, SpoT-T442I, SpoT-S434L, PolB-R477G, RpoC-H419P, RpoC-P64L, RpoB-K203T, Rnt-Q179P, Rnt-A27T, Rnt-F194L, Rnt-A180T, SapC-G79W; and/or a mutation which increases the expression of PyrE, such as, *e.g.* a mutation in *rph* or the *pyrE/rph* intergenic region.

The processes may further comprise

- a step of selecting any bacterial cell which has an improved tolerance to a diacid at a predetermined concentration in the medium, such as at least 1 g/L, such as at least 2 g/L, or

higher, such as at least 5 g/L or higher, such as at least 10 g/L or higher, such as at least 20 g/L or higher, such as at least 40 g/L or higher, such as at least 75 g/L or higher, such as at least 100 g/L or higher;

- an optional step of introducing a recombinant biosynthetic pathway for producing the diacid; or

- both of the above steps, in any order.

In one embodiment, the diacid is glutaric acid, and the predetermined concentration is at least 2 g/L or higher, such as at least 5 g/L or higher, such as at least 10 g/L or higher, such as at least 20 g/L or higher, such as at least 40 g/L or higher, such as at least 75 g/L or higher, such as at least 100 g/L or higher. In one embodiment, the diacid is adipic acid, and the predetermined concentration is at least 2 g/L or higher, such as at least 5 g/L or higher, such as at least 10 g/L or higher, such as at least 20 g/L or higher, such as at least 40 g/L or higher, such as at least 75 g/L or higher, such as at least 100 g/L or higher. In one embodiment, the diacid is pimelic acid, and the predetermined concentration is at least 2 g/L or higher, such as at least 5 g/L or higher, such as at least 10 g/L or higher, such as at least 20 g/L or higher, such as at least 30 g/L or higher, such as at least 45 g/L or higher, such as at least 75 g/L or higher. In one embodiment, the diacid is sebacic acid, and the predetermined concentration is at least 2 g/L or higher, such as at least 5 g/L or higher, such as at least 10 g/L or higher, such as at least 20 g/L or higher, such as at least 30 g/L or higher, such as at least 45 g/L or higher, such as at least 75 g/L or higher.

In a particular embodiment, the predetermined concentration is at most 20 g/L, such as at most 30 g/L, such as at most 50 g/L, such as at most 75 g/L, such as at most 100 g/L, such as at most 150 g/L.

Assays for assessing the tolerance of a modified bacterial cell to a diacid typically evaluate the growth rate, lag time, or both, of the bacterial cell at predetermined concentrations for the diacid in question, typically as compared to a control. Preferably, the control is the native or unmodified parent cell or strain, and an improved tolerance is identified as an improved growth rate, a reduced lag-time or both. For example, an improved growth rate can be at least 5%, such as at least 10%, such as at least 20%, such as at least 50%, such as at least 75% higher than that of the control, while a reduced lag time can be at least 10%, such as at least 20%, such as at least 50%, such as at least 75%, such as at least 90% shorter than that of the control. Specific assays are described, in detail, in the Examples.

Also provided is a method of producing a diacd, comprising culturing the bacterial cell obtained by any one of these methods, or the bacterial cell of any preceding aspect or embodiment, under conditions where the diacid is produced. Typically, these conditions include the presence of a suitable carbon source or mixes of different suitable carbon sources. Non-limiting examples of suitable carbon sources include, *e.g.*, sucrose, D-glucose, D-xylose, L-arabinose, glycerol; raw carbon feedstocks such as crude glycerol and cane syrup; as well as hydrolysates produced from cellulosic or lignocellulosic materials. For further details see, *e.g.*, Adkins *et al.*, 2013; Park *et al.*, 2013; Yu *et al.*, 2014; Cheong *et al.*, 2016; Deng and Mao, 2015; WO 2011/003034 A2 (Verdezyne); Curran *et al.*, 2013; Sengupta *et al.*, 2015; and Zhang *et al.*, 2015.

4) Compositions

A bacterial cell which has an increased tolerance to a diacid can be useful as a production host for the diacid. Bacterial cells according to the invention may have an increased growth rate, a decreased lag time, or both, in the diacid. For example, the bacterial cell may have Group 1 and/or Group 2 modifications providing for an increased growth rate, a reduced lag time, or both, of the cell in at least one diacid, *e.g.*, in glutaric acid, adipic acid, succinic acid, muconic acid, fumaric acid, itaconic acid, malic acid, malonic acid, maleic acid, glucaric acid, pimelic acid, suberic acid, sebacic acid, 2,5-furandicarboxylic acid, terephthalic acid, or azelaic acid, mesaconic acid, citraconic acid, tartaric acid, tartronic acid, diaminopimelic acid and/or glutaconic acid.

In one aspect, there is provided a composition comprising a plurality of bacterial cells according to any aspect or embodiment described herein, *e.g.*, an *in vitro* culture of such bacterial cells, optionally in a suitable culture medium and/or a chemically-defined medium comprising a carbon source. In one embodiment, the composition is substantially homogenous with respect to the bacterial cells.

In one aspect, there is provided a composition comprising a plurality of bacterial cells according to any preceding aspect or embodiment and a diacid. In one embodiment, the diacid is present at a concentration at which the genetic modification(s) and/or mutant(s) comprised in the bacterial cells results in an improved tolerance as compared to the parent bacterial cells, *e.g.*, wild-type or native bacterial cells. The concentrations at which bacterial cells according to the invention have improved tolerance are shown in Example 1, *e.g.*, in "Cross-compound tolerance testing". Typically, the concentration of the diacid is at least 1 g/L, such as at least 2 g/L, or higher, such as at least 5 g/L or higher, such as at least 10 g/L or higher, such as at least 20 g/L or higher, such as at least 40 g/L or higher, such as at least 45 g/L or higher, such as at least 75 g/L or higher, such as at least 100 g/L or higher.

In one aspect, there is provided a composition comprising

- a diacid at a concentration of at least 1 g/L, such as at least 2 g/L, or higher, such as at least 5 g/L or higher, such as at least 10 g/L or higher, such as at least 20 g/L or higher, such as at least 40 g/L or higher, such as at least 45 g/L or higher, such as at least 75 g/L or higher, such as at least 100 g/L or higher; and

- a plurality of bacterial cells according to any preceding aspect or embodiment.

In separate and specific embodiments, the diacid is glutaric acid, adipic acid, pimelic acid and sebacic acid, respectively. In other specific embodiments, the diacid present in the composition is at least 45 g/L fumaric acid, at least 45 g/L itaconic acid, at least 55 g/L malic acid, at least 50 g/L succinic acid, at least 45 g/L pimelic acid, and at least 38 g/L sebacic acid, respectively.

As described in Example 1; "Cross-compound tolerance testing," genetic modifications according to the invention also conferred tolerance to other chemicals, such as to other carboxylic acids (glutarate and adipate; hexanoate, octanoate, isobutyrate, glutarate and p-coumarate), to diamines (*e.g.*, HMDA, putrescine) and diols (2,3-butanediol, 1,2-propanediol). Accordingly, in one embodiment, there is provided a composition comprising a plurality of bacterial cells according to any preceding aspect or embodiment, and a chemical selected from the following, at at least the indicated concentration:

butanol	1.4% v/v
glutarate	40 g/L
p-coumarate	7.5 g/L
putrescine	32 g/L
HMDA	32 g/L
adipate	45 g/L
isobutyrate	7.5 g/L
hexanoate	3 g/L
octanoate	8 g/L
2,3-butanediol	6% v/v

1,2-propanediol	6% v/v
sodium chloride	0.6 M

Preferably, the bacterial cells are of the *Escherichia, Lactobacillus, Lactococcus, Bacillus, Pseudomonas, Corynebacterium, Deinococcus or Ralstonia* genera, such as, *e.g.*, *E. coli* cells, and comprise

a) at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*, or a combination of any thereof;

b) a mutation in one or more of SpoT, PolB, RpoC, RpoB, Rnt and SapC; an increased expression of one or more of SpoT, PolB, RpoC, RpoB, Rnt and SapC; and/or a mutation in *rph* or the *pyrE/rph* intergenic region which increases the expression of *pyrE*, or

c) a combination of (a) and (b).

Assays for assessing the tolerance of a modified bacterial cell to a selected diacid typically evaluate the growth rate, lag time, or both, of the bacterial cell at one or more predetermined concentrations of the compound, typically as compared to a control (*e.g.*, no compound). The predetermined concentrations(s) could be, for example, 1, 2, 5, 10, 20, 40, 45, 75, or 100 g/L. Preferably, the control is the native or unmodified parent cell or strain, and an improved tolerance is identified as an improved growth rate, a reduced lag-time or both. For example, an improved growth rate can be at least 5%, such as at least 10%, such as at least 20%, such as at least 50%, such as at least 75%, such as at least 100%, such as at least 200%, such as at least 300%, such as at least 500%, such as at least 1000%, such as at least 10000% higher than that of the control; while a reduced lag time can be at least 10%, such as at least 20%, such as at least 50%, such as at least 75%, such as at least 90% shorter than that of the control. Indeed, in some cases the native or unmodified parent cell cannot grow at all in a concentration of the diacid that the modified bacterial cell can grow in. Specific assays are described, in detail, in the Examples.

5) Bacterial cells

Also provided are strains, clones and other progeny of the bacterial cells of these and other aspects and embodiments, as well as cell cultures of such bacterial cells or strains. Typically, as used herein, a "strain" typically refers to a group of cells which are descendants of a initial

single colony of parent cells whereas a "clone" is a group of cells which are the descendants of an initial genetically modified single parent cell.

Non-limiting examples of bacterial cells suitable for modification according to any one of the aspects and embodiments described herein include bacteria of the *Escherichia, Lactobaccillus, Lactococcus, Corynebacterium, Bacillus, Ralstonia, Clostridia, Deinococcus* or *Pseudomonas* genera, such as from the *Escherichia, Lactobacillus, Lactococcus, Bacillus, Pseudomonas, Corynebacterium, Deinococcus or Ralstonia* genera. In one embodiment, the bacterial cell is an *E. coli* cell, such as a cell of the commercially available and/or fully characterized strains K-12 MG1655, BW25113, BL21, BL21(DE3), K-12 W3110, W, JM109, or Crooks (ATCC 8739). In a specific embodiment, the bacterial cell is derived from an *E. coli* K12 strain. In another embodiment, the bacterial cell is a *Lactobacillus* cell, such as a cell of the commercially available and/or fully characterized strains *Lactobacillus plantarum* JDM1, *Lactobacillus plantarum* WCFS1, and *Lactobacillus plantarum* NCIMB 8826. In another embodiment, the bacterial cell is a *Lactococcus* cell, such as a cell of the commercially available and/or fully characterized strains *Lactococcus lactis lactis* CV56, *Lactococcus lactis lactis* NIZO B40, and *Lactococcus lactis cremoris* NZ9000. In another embodiment, the bacterial cell is a *Bacillus* cell, such as a cell of the commercially available and/or fully characterized strains *Bacillus subtilis* 168 and *Bacillus subtilis* PY79. In one embodiment, the bacterial cell is a *Pseudomonas* cell, such as a cell of the commercially available and/or fully characterized strain *Pseudomonas putida* KT2440. In another embodiment, the bacterial cell is a *Ralstonia* cell, such as a cell of the commercially available and/or fully characterized strains *Ralstonia eutropha* H16 and *Ralstonia eutropha* JMP134. In another embodiment, the bacterial cell is a *Corynebacterium* cell, such as a cell of the commercially available and/or fully characterized strains 534 (ATCC 13032), K051, MB001, R, SCgG1, and SCgG2. In another embodiment, the bacterial cell is a *Deinococcus* cell, such as a *D. radiodurans* or *D. geothermalis* cell, such as a cell of the commercially available and/or fully characterized strain *D. radiodurans* R1.

While aspect and embodiments relating to bacterial cells herein typically refer to genes or proteins according to their designation in *E. coli*, for bacterial cells of another family or species, it is within the level of skill in the art to identify the corresponding gene or protein, *i.e.*, the ortholog and/or paralog, in the other family or species, typically by identifying sequences having moderate or high homology to the *E. coli* sequence, optionally taking the function of the protein expressed by the gene and/or the locus of the gene in the genome into account. Table 2 sets out the function of the protein encoded by each specific gene, the corresponding E.C. number (if applicable), its locus in the *E. coli* K-12 MG1655 genome and the SEQ ID number of the coding or non-coding sequence and, where applicable, the encoded amino acid sequence.

Tables 3 and 4 set out some examples of homologs or orthologs in selected organisms, identified in a preliminary and non-limiting analysis. Indeed, homologs or orthologs of these proteins exist also in other bacteria, and other homologs or orthologs not identified in this preliminary search can exist in the species listed in Table 3. The skilled person is well-familiar with different searching and/or screening methods for identifying homologs or orthologs across different species. To briefly summarize some of the preliminary findings in Table 3:

- KgtP, ProV, ProW, CspE, RfaE, PstS, pstA, pstB, pstC, UvrB, SpoT, RpoB, RpoC, SapC, and PyrE are widely conserved and were identified in all organisms. ProW and ProX appear to be fused in *Lactococcus lactis* and *Lactobacillus plantarum.*
- Two YbjL homologs or orthologs were identified in *Corynebacterium glutamicum.*
- Rph was found to be conserved in all organisms with the exception of *Lactococcus lactis*.
- ProQ was only found in *Pseudomonas putida*, indicating it is likely only conserved in the Gammaproteobacteria.
- RpoS is likely only conserved as an alternative sigma factor to RpoD in Gram-negative bacteria.
- SspA was found to be conserved in Gram-negative organisms.
- Tdk was found to be conserved between *E. coli* and only certain Gram-positive organisms in Table 3 (*B. subtilis, L. lactis, L. plantarum*)
- YcjG has a conserved annotated function in *Bacillus subtilis* and has homology to annotated muconate/chloromuconate cycloisomerases, which may be another activity of the enzyme, in the Gram-negative organisms (*P. putida, R. eutropha*) and *C. glutamicum.*
- PolB is conserved in the Gram-negative organisms (*P. putida, R. eutropha*)
- Rnt has a high degree of identity and with similar annotated functions to a protein in *P. putida*, and is a homolog of annotated DNA polymerases in *B. subtilis*, *L. plantarum*, *L. lactis*, and *R. eutropha*.

***Table 2**. Protein function and Locus IDs*

E. coli gene designation	Protein function	E.C. number	Locus ID	SEQ ID NO:
kgtP	α-ketoglutarate:H^+ symporter	N/A	b2587	1
ybjL	inner membrane protein YbjL	N/A	b0847	2
proV	glycine betaine/proline ABC	3.6.3.32	b2677	3

	transporter – ATP binding subunit			
proW	glycine betaine/proline ABC transporter – membrane subunit	3.6.3.32	b2678	4
proX	glycine betaine/proline ABC transporter – periplasmic binding protein	3.6.3.32	b2679	5
proQ	RNA chaperone, involved in posttranscriptional control of ProP levels	N/A	b1831	6
cspE	transcription antiterminator and regulator of RNA stability	N/A	b0623	7
rfaE	fused heptose-7-phosphate kinase/heptose-1-phosphate adenyltransferase	2.7.7.70, 2.7.1.167	b3052	8
yfbP	predicted protein	N/A	b2275	9
yfjM	CP4-57 prophage; predicted protein	N/A	b2629	10
pstS	phosphate ABC transporter – periplasmic binding protein	3.6.3.27	b3728	11
pstA	phosphate ABC transporter – membrane subunit	3.6.3.27	b3726	34
pstB	phosphate ABC transporter – ATP binding subunit	3.6.3.27	b3725	35
pstC	phosphate ABC transporter – membrane subunit	3.6.3.27	b3727	36
rph	RNAse PH	2.7.7.56	b3643	12
rpoS	RNA polymerase, sigma S (sigma 38) factor	N/A	b2741	13
sspA	stringent starvation protein A	N/A	b3229	14
tdk	thymidine kinase/deoxyuridine kinase	2.7.1.145, 2.7.1.21	b1238	15
uvrB	DNA repair; excision nuclease subunit B	3.1.25.-	b0779	16
ycjG	L-Ala-D/L-Glu epimerase	5.1.1.20	b1325	17
yeaR	conserved protein	N/A	b1797	18

spoT	guanosine 3'-diphosphate 5'-triphosphate 3'-diphosphatase [multifunctional]	3.1.7.2	b3650	19 (DNA) 20 (protein)
polB	DNA polymerase II	3.1.11.-, 2.7.7.7	b0060	21 (DNA) 22 (protein)
rpoB	RNA polymerase, β subunit	2.7.7.6	b3987	23 (DNA) 24 (protein)
rpoC	RNA polymerase, β' subunit	2.7.7.6	b3988	25 (DNA) 26 (protein)
rnt	RNase T	3.1.13.-	b1652	27 (DNA) 28 (protein)
sapC	integral membrane protein SapC of predicted ABC transporter	N/A	b1292	29 (DNA) 30 (protein)
pyrE	Orotate phosphoribosyltransferase	2.4.2.10	b3642	31 (DNA) 32 (protein)
pyrE/rph intergenic region	-	-	-	33

Table 3. *Homologs or orthologs identified by protein BLAST (BLASTP) of E. coli K-12 MG1655 proteins against protein databases from selected reference organisms. Hits with the largest e-value are shown, and hits are only shown when the e-value < 1.0. Hit proteins with e-value < 0.1 (non-italicized) are deemed more probable of having the same or similar function as the E. coli protein.*

Protein (# of residues)	*B. subtilis* 168	*P. putida* KT2440	*L. plantarum* JDM1	*L. lactis* KF147	*Ralstonia eutropha* H16	*Corynebacterium glutamicum* ATCC 13032
KgtP (432 aa)	24-27% identity (339-420 aa) "metabolite transporter" (NP_388707.1), "metabolite transport protein YwtG" (NP_391464.2), "metabolite transport protein YncC" (NP_389645.2), "major myo-inositol transporter IolT" (NP_388504.1)	56-70% identity (415-416 aa) "major facilitator superfamily metabolite/H+ symporter" (NP_743537.1, NP_743559.1)	23-28% identity (176-355 aa) "arabinose transport protein" (YP_003064425.1), "transport protein" (YP_003063609.1), "sugar transport protein" (YP_003064460.1)	23% identity (347 aa) "arabinose-proton symporter" (NP_003354045.1)	33-37% identity (337-381 aa) "major facilitator superfamily transporter MHS family protein" (YP_725964.1, YP_725210.1), "MFS family transporter" (YP_725057.1)	28-33% identity (392-443 aa) "integral membrane transport protein" (NP_602106.1), "proline-betaine transporter" (NP_602258.1), "major facilitator superfamily permease" (NP_599668.1, NP_599535.2, NP_600684.1)
YbjL (561 aa)	*28% identity (116 aa) "sulfate transporter YvdB"* (NP_391346.1)	31% identity (123 aa) "potassium/proton antiporter" (NP_747167.2)	*34% identity (68 aa) "sugar transport protein"* (YP_003064460.1)	*25% identity (76 aa) "chloride channel protein"* (YP_003354224.1)	31% identity (566 aa) "aspartate: alanine antiporter" (YP_726110.1); 31% identity (550 aa) "permease" (YP_725451.1)	25% identity (548 aa) "permease" (NP_601414.1); 25% identity (550 aa) "hypothetical protein NCgl0565" (NP_599826.1)
ProV (400 aa)	51% identity (390 aa) "glycine/betaine ABC transporter ATP-binding protein" (NP_388180.2)	36-51% identity (222-352 aa) "glycine betaine/L-proline ABC transporter ATP-binding subunit" (NP_742461.1), "glycine betaine/L-proline ABC transporter ATPase/permease fusion protein" (NP_744918.1), "glycine betaine/L-proline ABC transporter ATPase" (NP_743029.1)	34-44% identity (224-361 aa) "glycine/betaine/carnitine/choline ABC transporter ATP-binding protein" (YP_003062931.1, YP_003061916.1)	39-48% identity (247-392 aa) "glycine betaine/carnitine/choline ABC transporter ATP-binding protein" (YP_003353988.1), "glycine/betaine ABC transporter ATP-binding protein" (YP_003353316.1)	33-44% identity (195-354 aa) "ABC transporter ATPase" (YP_724876.1, YP_726702.1, YP_725457.1, YP_725326.1, YP_726845.1, YP_724565.1, YP_727745.1, YP_727463.1, YP_725974.1, YP_724993.1, YP_727203.1, YP_725812.1, YP_726707.1), "ABC-type transporter, ATPase component" (YP_727764.1)	35-40% identity (226-283 aa) "ABC transporter ATPase" (NP_599870.1, NP_601662.1, NP_599673.1, NP_599959.1, NP_600605.1, NP_600550.1), "glutamate ABC transporter ATPase" (NP_601157.1), "ABC transporter duplicated ATPase" (NP_601199.1)

ProW (354 aa)	48% identity (275 aa) "glycine betaine transport system permease protein OpuAB" (NP_388181.1)	40-53% identity (206-265 aa) "glycine betaine/L-proline ABC transporter permease" (NP_742462.1), "binding protein-dependent transport system inner membrane protein" (NP_745696.1), "glycine betaine/L-proline ABC transporter ATPase/permease fusion protein" (NP_744918.1)	34-40% identity (161-169 aa) "glycine betaine/carnitine/ choline ABC transporter, substrate binding and permease protein" (YP_003061915.1), "glycine betaine/carnitine/ choline ABC transporter, permease protein" (YP_003062932.1)	35-47% identity (155-284 aa) "glycine betaine/carnitine/ choline ABC transporter permease/substrate-binding protein" (YP_003353987.1), "glycine betaine ABC transporter permease/substrate-binding protein (YP_003353317.1)	27-34% identity (152-194 aa) "ABC transporter permease" (YP_725456.1, YP_725454.1, YP_726844.1, YP_724987.1), "ABC-type transporter, fused periplasmic and permease components" (YP_726088.1)	25-30% identity (157-232 aa) "ABC transporter permease" (NP_600676.1, NP_600445.1)
ProX (330 aa)	25% identity (124 aa) "glycine betaine-binding protein OpuAC" (NP_388182.1)	21-27% identity (155-327 aa) "glycine betaine ABC transporter substrate-binding protein" (NP_745695.1, NP_744919.1), "glycine/betaine-binding protein" (NP_742246.1)	Not found	22% identity (279 aa) "glycine betaine/carnitine/ choline ABC transporter permease/substrate-binding protein" (YP_003353987.1)	*29% identity (75 aa) "RND superfamily exporter"* (YP_725351.1)	Not found
ProQ (232 aa)	*24% identity (128 aa) "hypothetical protein BSU32070"* (NP_391087.1)	27% identity (115 aa) "ProQ activator of osmoprotectant transporter ProP" (NP_744331.1)	Not found	Not found	*29% identity (76 aa) "dehydrogenase"* (YP_724959.1)	*33% identity (66 aa) "elongation factor Ts"* (NP_601230.1)
CspE (69 aa)	61-68% identity (62-64 aa) "cold shock protein CspB" (NP_388791.1), "cold shock protein CspC" (NP_388393.1), "cold shock protein CspD" (NP_390076.1)	54-62% identity (61-68 aa) "cold shock protein CspA" (NP_743679.1), "cold-shock domain-contain protein" (NP_743146.1, NP_743260.1), "cold shock protein CspA" (NP_744611.1), "cold-shock domain-contain protein, partial" (NP_743369.1), "cold-shock protein CspD" (NP_746140.1)	65-69% identity (61-62 aa) "cold shock protein CspP" (YP_003062538.1), "cold shock protein CspL" (YP_003061635.1, YP_003061614.1), "cold shock protein CspC" (YP_003062410.1)	62-66% identity (61-62 aa) "cold-shock protein" (YP_003352748.1, YP_003353646.1, YP_003352635.1, YP_003354678.1)	62% identity (61 aa) "cold shock protein, DNA-binding" (YP_727497.1)	63-65% identity (65 aa) "cold shock protein" (NP_599426.1, NP_599560.1)

RfaE (477 aa)	32% identity (133 aa) "glycerol-3-phosphate cytidylyltransferase", 24-26% identity (228-327 aa) "ribokinase" (NP_391473.1), "sugar kinase YdjE" (NP_388498.1), "fructosamine kinase FrlD" (NP_391137.1)	57% identity (474 aa) "bifunctional heptose-7-phosphate kinase/heptose-1-phosphate adenyltransferase" (NP_747037.1)	34% identity (128 aa) "glycerol-3-phosphate cytidylyltransferase" (YP_003062640.1), 24% identity (240-309 aa) "ribokinase" (YP_003064511.1, YP_003063470.1)	35% identity (98 aa) "glycerol-3-phosphate cytidylyltransferase" (YP_003352686.1); 33% identity (147 aa) "glycerol-3-phosphate cytidyltransferase" (YP_003353407.1)	49% identity (312 aa) "D-beta-D-heptose 7-phophosphate kinase" (YP_725318.1)	28% identity (120 aa) "ribokinase sugar kinase" (NP_599410.1)
YfbP (282 aa)	*28% identity (68 aa) "sensor histidine kinase"* (NP_391844.1)	*24% identity (148 aa) "tryptophan synthase subunit alpha"* (NP_742252.1)	Not found	*24% identity (106 aa) "multimodular transpeptidase-transglycosylase Pbp2A"* (YP_003354748.1)	*29% identity (106 aa) "O-linked N-acetylglucosamine transferase OGT"* (YP_724894.1)	33% identity (39 aa) "hypothetical protein NCgl0374" (NP_599633.1)
YfjM (87 aa)	Not found	*30% identity (54 aa) "ABC transporter substrate-binding protein"* (NP_744413.1)	*26% identity (53 aa) "small heat shock protein"* (YP_003064271.1)	Not found	Not found	Not found
PstS (346 aa)	26% identity (229 aa) "phosphate-binding protein PstS" (NP_390378.1)	39% identity (339 aa) "phosphate ABC transporter substrate-binding protein" (NP_744800.1)	27-28% identity (224-318 aa) "phosphate ABC transporter substrate-binding protein" (YP_003062200.1, YP_003062190.1)	26% identity (232-251 aa) "phosphate ABC transporter substrate-binding protein" (YP_003354292.1, YP_003354291.1)	64% identity (347 aa) "ABC transporter periplasmic protein" (YP_726901.1)	30% identity (282 aa) "ABC transporter periplasmic component" (NP_601773.1)
Rph (228 aa)	58% identity (222 aa) "ribonuclease PH" (NP_390715.1)	69% identity (228 aa) "ribonuclease PH" (NP_747395.1)	Not found	24-27% identity (59-207 aa in stretches) "polyribo-nucleotide nucleotidyltrans-ferase" (YP_003354448.1)	62% identity (221 aa) "ribonuclease PH" (YP_725462.1)	59% identity (217 aa) "ribonuclease PH" (NP_601703.2)
RpoS (330 aa)	43% identity (321 aa) "RNA polymerase sigma factor RpoD" (NP_390399.2)	76% identity (277 aa) "RNA polymerase sigma factor RpoS" (NP_743780.1)	44% identity (271 aa) "RNA polymerase sigma factor RpoD" (YP_003063237.1)	40% identity (288 aa) "RNA polymerase sigma factor RpoD" (YP_003352999.1)	52% identity (280 aa) "RNA polymerase sigma factor RpoS" (YP_726836.1)	39% identity (316 aa) "RNA polymerase sigma factor SigB" (NP_601125.1), 36% identity (304 aa) "RNA polymerase sigma factor" (NP_601117.2)
SspA (212 aa)	Not found	57% identity (200 aa) "stringent starvation protein A" (NP_743480.1)	*45% identity (22 aa) "hypothetical protein JDM1_0823"* (YP_003062407.1)	Not found	46% identity (203 aa) "stringent starvation protein A" (YP_727831.1)	*56% identity (16 aa) "hypothetical protein NCgl2333"* (NP_601617.1)
Tdk (205 aa)	30% identity (184 aa) "thymidine kinase" (NP_391587.1)	Not found	45% identity (152 aa) "thymidine kinase" (YP_003063573.1)	49% identity (187 aa) "thymidine kinase" (YP_003353039.1)	*25% identity (132 aa) "prolyl-tRNA synthetase"* (YP_727689.1)	Not found

UvrB (673 aa)	59% identity (666 aa) "UvrABC system protein B" (NP_391397.1)	69% identity (670 aa) "excinuclease ABC subunit B" (NP_744125.1)	59% identity (663 aa) "excinuclease ABC subunit B" (YP_003062224.1)	55% identity (693 aa) "excinuclease ABC subunit B" (YP_003353008.1)	66% identity (673 aa) "excinuclease ABC subunit B" (YP_725661.1)	56% identity (668 aa) "excinuclease ABC subunit B" (NP_600587.1)
YcjG (321 aa)	31% identity (322 aa) "L-Ala-D/L-Glu epimerase" (NP_389181.1)	26% identity (337 aa) "muconate and chloromuconate cycloisomerase" (NP_745848.1)	*31% identity (91 aa) "phosphopyruvate hydratase"* (YP_003063198.1)	24% identity (338 aa) "O-succinylbenzoate synthase" (YP_003353203.1)	26% identity (324 aa) "muconate cyclo-isomerase" (YP_726435.1)	27% identity (290 aa) "chloromuconate cycloisomerase" (NP_601602.2)
YeaR (119 aa)	*26% identity (91 aa) "hypothetical protein BSU13060"* (NP_389189.1)	*24% identity (41 aa) "30S ribosomal protein S12"* (NP_742615.1)	Not found	Not found	Not found	Not found
SpoT (702 aa)	40% identity (719 aa) "GTP pyrophosphokinase" (NP_390638.2)	37-55% identity (681-701 aa) "(p)ppGpp synthetase I SpoT/RelA" (NP_747403.1, NP_743813.1)	38% identity (741 aa) "GTP pyrophosphokinase" (YP_003063260.1)	40% identity (725 aa) "GTP pyrophosphokinase/ guanosine-3,5-bis(diphosphate) 3-pyrophospho-hydrolase" (YP_003352549.1)	36-47% identity (674-720 aa) "GTP pyrophosphokinase" (YP_725468.1, YP_725845.1)	38% identity (723 aa) "guanosine polyphosphate pyrophospho-hydrolase/ synthetase" (NP_600866.1)
PolB (783 aa)	Not found	68% identity (784 aa) "DNA polymerase II" (NP_744541.1)	Not found	*32% identity (63 aa) "O-succinylbenzoate synthase"* (YP_003353203.1)	68% identity (784 aa) "DNA polymerase II" (YP_726151.1)	*35% identity (55 aa) "rhodanese-related sulfurtransferase"* (NP_599306.1)
RpoB (1342 aa)	59% identity (533 aa) "DNA-directed RNA polymerase subunit beta" (NP_387988.2)	72% identity (1360 aa) "DNA-directed RNA polymerase subunit beta" (NP_742613.1)	47-52% identity (304-953 aa in stretches) "DNA-directed RNA polymerase subunit beta" (YP_003062426.1)	46-47% identity (307-951 aa in stretches) "DNA-directed RNA polymerase subunit beta" (YP_003354373.1)	66% identity (1370 aa) "DNA-directed RNA polymerase subunit beta" (YP_727933.1)	41-56% identity (238-616 aa in stretches) "DNA-directed RNA polymerase subunit beta" (NP_599733.1)
RpoC (1407 aa)	50% identity (1134 aa) "DNA-directed RNA polymerase subunit beta'" (NP_387989.2)	75% identity (1399 aa) "DNA-directed RNA polymerase subunit beta'" (NP_742614.1)	44-51% identity (235-1061 aa in stretches) "DNA-directed RNA polymerase subunit beta'" (YP_003062427.1)	48-51% identity (238-1043 aa in stretches) "DNA-directed RNA polymerase subunit beta'" (YP_003354372.1)	67% identity (1397 aa) "DNA-directed RNA polymerase subunit beta'" (YP_727932.1)	46-50% identity (206-819 aa in stretches) "DNA-directed RNA polymerase subunit beta'" (NP_599734.1)
Rnt (215 aa)	27% identity (191 aa) "DNA polymerase III PolC-type" (NP_389540.1)	63% identity (198 aa) "Ribonuclease T" (NP_743246.1)	28% identity (180 aa) "DNA polymerase III PolC" (YP_003063293.1)	29% identity (206 aa) "DNA polymerase III subunit alpha" (YP_003354765.1)	27% identity (180 aa) "DNA polymerase III subunit epsilon" (YP_726924.1)	Not found

SapC (296 aa)	28-30% identity (286-288 aa) "oligopeptide transport system permease protein AppC" (NP_389022.1), "dipeptide transport system permease protein DppC" (NP_389177.1), "oligopeptide transport system permease protein OppC" (NP_389027.1)	42% identity (281 aa) "binding-protein-dependent transport system inner membrane protein" (NP_743041.1)	25% identity (302 aa) "peptide ABC transporter permease" (YP_003062653.1)	23-27% identity (219-306 aa) "peptide ABC transporter permease" (YP_003354430.1, YP_003352873.1)	34-37% identity (216-238 aa) "ABC transporter permease" (YP_725975.1, YP_726565.1, YP_726551.1, YP_727395.1)	31-38% identity (215-230 aa) "ABC transporter permease" (NP_601522.1, NP_601635.1, NP_601198.1)
PyrE (213 aa)	25-34% identity (in stretches) "orotate phosphoribosyl -transferase" (NP_389439.1)	67% identity (213 aa) "orotate phosphoribosyl-transferase" (NP_747392.1)	29% identity (138 aa) "orotate phosphoribosyl-transferase" (YP_003063746.1)	30% identity (131 aa) "orotate phosphoribosyl-transferase" (YP_003353548.1)	56% identity (215 aa) "orotate phosphoribosyl-transferase" (YP_724744.1)	29% identity (139 aa) "orotate phosphoribosyl-transferase" (NP_601967.1)

Table 4. *Homologs or orthologs identified by protein BLAST (BLASTP) of E. coli K-12 MG1655 proteins against protein databases from the two chromosomes and two endogenous plasmids of Deinococcus radiodurans R1. Hits with the largest e-values are shown, and hits are only shown when the e-value < 1.0.*

Protein (# of residues)	***D. radiodurans* R1 chromosome 1 (NC_001263)**	***D. radiodurans* R1 chromosome 2 (NC_001264)**	***D. radiodurans* R1 circular plasmid 1 (NC_000959)**	***D. radiodurans* R1 megaplasmid 1 (NC_000958)**
KgtP (432 aa)	36% identity (56 aa) "hypothetical protein DR_1056" (NP_294780.1)	30% identity (107 aa) "sugar transporter putative" (NP_285594.1)	32% identity (73 aa) "hypothetical protein DR_C0021" (NP_051691.1)	58% identity (17 aa) "hypothetical protein DR_B0098" (NP_051631.1)
YbjL (561 aa)	24% identity (169 aa) "sodium/sulfate symporter family protein" (NP_295134.1)	33% identity (52 aa) "transcriptional regulator" (NP_285659.1)	Not found	Not found
ProV (400 aa)	35-41% identity (196-265 aa) "spermidine/putrescine ABC transporter ATP-binding protein" (NP_295026.1), "ABC transporter ATP-binding protein" (NP_295079.1, NP_295920.1, NP_293788.1), "amino acid ABC transporter ATP-binding protein" (NP_295371.1), "sugar ABC transporter, ATP-binding protein" (NP_295876.1), "peptide ABC transporter ATP-binding protein" (NP_295290.1)	27-38% identity (215-317 aa) "amino acid ABC transporter ATP-binding protein" (NP_285461.1), "phosphate ABC transporter ATP-binding protein" (NP_285484.1), "branched-chain amino acid ABC transporter ATP-binding protein" (NP_285584.1), "ABC transporter ATP-binding protein" (NP_285331.1, NP_285672.1), "urea/short-chain amide ABC transporter ATP-binding protein" (NP_285647.1)	36% identity (28 aa) "transposase-related" (NP_051690.1)	32% identity (186-231 aa) "iron ABC transporter ATP-binding protein" (NP_051651.1), "ABC transporter, ATP-binding protein" (NP_051588.1)
ProW	26-31% identity (116-183 aa)	30-35% identity (114-158	29% identity (62 aa)	28% identity (57 aa) "iron

(354 aa)	"ABC transporter ATP-binding protein" (NP_294234.1), "ABC transporter permease" (NP_295919.1)	aa) "amino acid ABC transporter permease" (NP_285462.1, NP_285460.1)	"transposase, putative" (NP_051699.1)	ABC transporter permease" (NP_051652.1)
ProX (330 aa)	Not found	29% identity (68 aa) "putative FAD-binding dehydrogenase" (NP_285561.1)	29% identity (41 aa) "phosphoenolpyruvate synthase-related protein" (NP_051677.1)	65% identity (17 aa) "hypothetical protein DR_B0023" (NP_051564.1)
ProQ (232 aa)	31% identity (58 aa) "hypothetical protein DR_2622" (NP_296341.1)	Not found	Not found	Not found
CspE (69 aa)	59% identity (64 aa) "CSD family cold shock protein" (NP_294631.1)	36-39% identity (31-36 aa) "methyl-accepting chemotaxis-like protein" (NP_285676.1), "methyl-accepting chemotaxis protein" (NP_285677.1)	22-33% identity (45-50 aa) "hypothetical protein DR_C0022" (NP_051692.1), "nodulation protein-related protein" (NP_051705.1)	Not found
RfaE (477 aa)	26% identity (274 aa) "carbohydrate kinase" (NP_296273.1)	33% identity (78 aa) "ribokinase" (NP_285378.1)	30% identity (54 aa) "coenzyme PQQ synthesis protein, putative" (NP_051702.1)	26% identity (248 aa) "1-phosphofructokinase" (NP_051610.1)
YfbP (282 aa)	Not found	39% identity (61 aa) "hypothetical protein DR_A0109" (NP_285432.1)	21% identity (85 aa) "oxidative cyclase, putative" (NP_051704.1)	Not found
YfjM (87 aa)	Not found	Not found	Not found	Not found
PstS (346 aa)	Not found	44% identity (336 aa) "phosphate ABC transporter periplasmic phosphate-binding protein" (NP_285481.1)	Not found	36% identity (47 aa) "hypothetical protein DR_B0023" (NP_051564.1)
Rph (228 aa)	44% identity (215 aa) "ribonuclease PH" (NP_295308.1)	33% identity (62 aa) "uroporphyrin-III C-methyltransferase/ uroporphyrinogen-III synthase (NP_285335.1)	Not found	35% identity (31 aa) "iron-chelator utilization protein" (NP_051560.1)
RpoS (330 aa)	42% identity (288 aa) "RNA polymerase sigma-A factor" (NP_294640.1)	30% identity (47 aa) "hypothetical protein DR_A0192" (NP_285515.1)	34% identity (50 aa) "hypothetical protein DR_C0027" (NP_051697.1)	Not found
SspA (212 aa)	24-29% identity (48-99 aa) "glutaredoxin" (NP_295808.1), "hypothetical protein DR_0390" (NP_294113.1)	35% identity (46 aa) "P49 secreted protein" (NP_285686.1)	24% identity (71 aa) "hypothetical protein DR_C0009" (NP_051682.1)	Not found
Tdk (205 aa)	27% identity (191 aa) "thymidine kinase" (NP_295707.1)	24% identity (80 aa) "urea/short-chain amide ABC transporter periplasmic urea/short-chain amide-binding protein" (NP_285643.1)	27% identity (52 aa) "putative transposase" (NP_277100.1)	38% identity (50 aa) "ABC transporter, ATP-binding protein" (NP_051588.1)
UvrB (673 aa)	56% identity (661 aa) "excinuclease ABC subunit B" (NP_295996.1)	33% identity (96 aa) "hypothetical protein DR_A0131" (NP_285455.1)	Not found	Not found
YcjG (321 aa)	24-30% identity (285-306 aa) "chloromuconate cyclosisomerase" (NP_295594.1), "N-acylamino acid racemase" (NP_293770.1)	Not found	Not found	29% identity (201 aa) "N-acylamino acid racemase" (NP_051613.1)
YeaR (119 aa)	Not found	37% identity (35 aa) "exopolyphosphatase" (NP_285509.1)	29% identity (68 aa) "nodulation protein-related protein" (NP_051705.1)	30% identity (89 aa) "KdpD-related protein" (NP_051621.1)

SpoT (702 aa)	38% identity (734 aa) "GTP pyrophosphokinase" (NP_295561.1)	30% identity (53 aa) "long-chain-fatty-acid—CoA ligase" (NP_296364.1)	Not found	Not found
PolB (783 aa)	24-32% identity (65-135 aa) "excinuclease ABC subunit A" (NP_295494.1), "hypothetical protein DR_2521" (NP_296241.1)	34% identity (65 aa) "P49 secreted protein" (NP_285686.1)	54% identity (13 aa) "hypothetical protein DR_C0014" (NP_051686.1)	30% identity (69 aa) "hypothetical protein DR_B0054" (NP_051592.1)
RpoB (1342 aa)	40-53% identity (in stretches, 227-620 aa) "DNA-directed RNA polymerase subunit beta" (NP_294636.1)	29% identity (63 aa) "hypothetical protein DR_A0017" (NP_285341.1)	33% identity (40 aa) "modification methylase, putative" (NP_277101.1)	34% identity (44 aa) "hypothetical protein DR_B0144" (NP_051673.1)
RpoC (1407 aa)	45-58% identity (in stretches, 156-937 aa) "DNA-directed RNA polymerase subunit beta'" (NP_294635.1)	37% identity (30 aa) "succinate-semialdehyde dehydrogenase" (NP_285327.1)	48% identity (27 aa) "putative transposase" (NP_277100.1)	32% identity (62 aa) "hypothetical protein DR_B0013" (NP_051556.1)
Rnt (215 aa)	25% identity (192 aa) "DNA polymerase III subunit epsilon" (NP_294580.1)	38% identity (37 aa) "transcriptional regulator" (NP_285659.1)	22% identity (79 aa) "hypothetical protein DR_C0027" (NP_051697.1)	Not found
SapC (296 aa)	26-39% identity (215-230 aa) "peptide ABC transporter permease" (NP_294682.1, NP_295292.1, NP_294088.1)	32% identity (248 aa) "peptide ABC transporter permease" (NP_285531.1)	Not found	39% identity (23 aa) "sensor histidine kinase, copper metabolism" (NP_051623.1)
PyrE (213 aa)	31% identity (154 aa) "orotate phosphoribosyltransferase" (NP_294170.1)	32% identity (86 aa) "serine protease" (NP_285606.1)	Not found	38% identity (39 aa) "hypothetical protein DR_B0068" (NP_051604.1)

So, in one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein each recited gene is instead (i) a gene encoding the corresponding (homolog or ortholog) protein in Table 3 or 4 (ii) a gene located at the corresponding locus, or (iii) both.

EXAMPLE 1

Methods

Screening for tolerance in wild-type cells

Escherichia coli K-12 MG1655 was grown overnight in M9 minimal medium + 1% glucose and subcultured the following morning to an initial OD_{600} of 0.05 in M9 + 1% glucose. Cells were grown to mid-exponential phase (OD_{600} 0.7-1.0) and were back-diluted with fresh medium to an OD_{600} of 0.7. The diluted cells were used to inoculate M9 + 1% glucose containing varying concentrations of glutaric acid or adipic acid which were neutralized to pH 7.0 with sodium hydroxide, and growth was measured in FlowerPlates in a Biolector microbioreactor system (m2p-labs) at 37°C with 1000 rpm shaking. The culture volume in each well was 1.4 mL.

Adaptive laboratory evolution of tolerant strains

Based on the screening results, *E. coli* K-12 MG1655 was grown overnight in M9 minimal medium and 150 µL was transferred the next day into 8 tubes containing 15 mL of M9 + 1% glucose + 20 g/L glutaric acid or 25 g/L adipic acid on a Tecan Evo robotic platform custom-designed for performing adaptive laboratory evolutions (ALE). Cells were cultured on a 37°C heat block with stirring by magnetic stir bars. Culture OD_{600} was monitored at times determined by a predictive custom script, and when the OD_{600} reached approximately 0.3, 150 µL of culture was inoculated into a new tube with the same media concentration. Instrument downtime would occasionally result in cells overgrowing to saturation or an OD_{600} greater than 0.3, and reinoculations were occasionally performed from cryogenic stocks of the population. When the growth rate was observed to substantially increase, the media concentration was changed. These concentration changes for glutaric acid were to 30 g/L, 40 g/L, and 45 g/L, and 47.5 g/L, while the changes adipic acid were to 35 g/L, 40 g/L, 45 g/L and 50 g/L. Approximately 100 µL of each population (8 per chemical) were plated on LB agar and incubated at 37°C overnight.

Primary screening of ALE isolates

Five colonies from wild-type K-12 MG1655 and 10 individual colonies deriving from each population were inoculated into 300 µL M9 + 1% glucose in 96 well deepwell plates and incubated in a 300 rpm plate shaker at 37°C. The next day, cells were diluted 10X in M9 + 1% glucose and 30 µL was transferred into clear-bottomed 96 well half-deepwell plates (with rectangular wells) containing M9 + 1% glucose and M9 + 1% glucose + 52.78 g/L glutaric acid or 55.56 g/L adipic acid, such that the final concentration of glutaric acid or adipic acid was 47.5 g/L or 50 g/L, respectively. In addition, cryogenic glycerol stocks of the overnight culture were saved in a 96 well plate format. Half deepwell plates were incubated at 37°C with 225 rpm shaking in a Growth Profiler (Enzyscreen), with optical scans of the plates taken at 15 minute intervals. Green pixel values integrated over a 1 mm diameter circular area in each well were converted to OD_{600} values using a previously determined calibration between OD_{600} and green pixel values. Resulting growth curves were visually inspected for isolates exhibiting the most robust or unique growth patterns within each population. In general, it was attempted to select three isolates per population for further analysis, and all populations were represented in the resequenced isolates.

Secondary screening of ALE isolates

Selected isolates from the primary screen were restruck onto LB agar from the cryogenic stock made from the overnight culture plate for the primary screen. Five K-12 MG1655 colonies and three individual colonies from each isolate were inoculated as biological replicates into a new 96 well deepwell plate containing 300 µL of M9 + 1% glucose, and

grown overnight as for the primary screen. The next day, a cryogenic stock and half deepwell plates containing M9 + 1% glucose with or without glutaric acid or adipic acid were inoculated using the plate of overnight cultures, and growth was measured as described for the primary screen. Resulting growth curves were visually inspected for isolates exhibiting robust and reproducible growth between replicates in high concentrations of glutaric acid or adipic acid.

Re-sequencing of ALE isolates

A total of 20 isolates were selected from the secondary screen for whole-genome resequencing. An individual colony was taken from the LB agar plates prepared following the primary screen, inoculated into 2 mL LB, and grown overnight at 37°C in a 250 rpm shaker. The following morning, 0.5 mL of cells were transferred to microcentrifuge tubes and centrifuged at 16000 x *g* for 2 minutes. The supernatant was removed and pellets were stored at -20°C until further processing. Genomic DNA was extracted from thawed cell pellets using a PureLink genomic DNA extraction kit, with further concentration and purification performed by ethanol precipitation. To generate libraries for sequencing, the Illumina TruSeq Nano kit was used according to the manufacturers' directions using an input quantity of 200 ng of genomic DNA from each isolate. Sequencing was performed on an Illumina MiSeq sequencer, with a minimum 20X average genomic coverage ensured for each isolate based on the number of reads. Fastq output files were analyzed for variants compared to the K-12 MG1655 reference genome (accession number NC_000913.3) using breseq (www-adress barricklab.org/twiki/bin/view/Lab/ToolsBacterialGenomeResequencing).

Sole carbon source plate growth assay

M9 agar plates lacking glucose and instead containing 10 g/L of glutaric acid or 10 g/L of adipic acid (both neutralized to pH 7.0 with sodium hydroxide) were prepared, and strains were struck onto wedges of the plate from a colony on an LB plate. Plates were incubated for up to 4 weeks at 37°C.

Construction of gene knockouts

Probable important losses-of-function were determined by identifying genes across all isolates that harboured mutations, especially those occurring in multiple populations, and by the presence of at least one mutation that either generated a premature stop codon, a frameshift mutation, or the presence of an insertion element sequence within the gene. For those genes, the corresponding knockout strain from the Keio collection of single knockout mutants (where each gene is replaced with a cassette consisting of a kanamycin resistance gene flanked by FRT sites) was used as a donor strain for P1*vir* phage transduction. Briefly, the Keio strain was grown to early exponential phase in LB + 5 mM $CaCl_2$ and 80 µL of a

P1*vir* stock raised on K-12 MG1655 was added. After significant lysis was observed after 1.5 to 2 hours, the lysate was filter-sterilized to remove cells and stored at 4°C. Strain K-12 MG1655 was grown overnight in LB + 5 mM $CaCl_2$ and 100 µL of the overnight culture was mixed with 100 µL of the P1*vir* lysate of the Keio collection mutant, and the mixture was incubated at 37°C without shaking for 20 minutes. The entire mixture was then plated on LB agar containing 1.25 mM sodium pyrophosphate as a chelating agent and 25 µg/mL kanamycin. One colony was then restruck on LB + 1.25 mM $Na_2P_4O_7$ + 25 µg/mL kanamycin plate and analyzed for presence of the Keio cassette in place of the wild-type gene by colony PCR. When further knockouts were constructed in the same strain, the Keio cassette was flipped out to generate a scar sequence such that Kan^R marker could be recycled. This was performed by transforming with pCP20, which constitutively expresses a flippase recombinase, and plating cells on LB agar + 100 µg/mL ampicillin and incubating at 30°C. The next day, one or more colonies was tested by colony PCR for loss of the Keio cassette, and successful mutants were then cured of pCP20 by elevated temperature curing at 40°C. Strains were verified to be cured of plasmid by plating on LB agar + 100 µg/mL ampicillin and incubation at 30°C. P1*vir* transductions were then performed using these mutant strains as recipients.

Biolector growth screening of evolved isolates and reconstructed mutants

Biological triplicate cultures of each strain were grown to saturation overnight in 96 well deepwell plates containing 300 µL M9 + 1% glucose. The next day, cells were diluted 1:10 in deionized water in a clear 96 well plate and the OD_{600} was measured on a BioTek plate reader. 48 well FlowerPlates containing a final volume of 1.4 mL of M9 + 1% glucose (plus relevant chemical) were inoculated to OD_{600} 0.03 (with plate reader pathlength, 200 µL volume) with the overnight culture and sealed with Breathseal film. Light backscatter intensity was monitored in a Biolector microbioreactor system at 37°C with 1000 rpm shaking.

Keio collection screening for loss-of-function mutations

For primary screening, Keio collection mutants were inoculated directly from a cryogenic stock of the Keio collection into 300 µL LB medium containing 25 µg/mL kanamycin in 96 well deepwell plates and grown at 37°C with 300 rpm shaking overnight. The Keio background strain, BW25113, was also inoculated into wells of this plate as a control. A cryogenic stock was made from each plate, and the cryogenic stock was replica plated into another 96 well deepwell plate containing 300 µL M9 + 1% glucose and grown overnight. The next day, cells were inoculated 1:100 into clear bottomed 96 well half-deepwell plates containing M9 + 1% glucose plus 40 g/L and 47.5 g/L putrescine, or 45 and 50 g/L adipic acid, and cultivated in a Growth Profiler as previously described for screening of ALE isolates.

As a secondary screen, promising Keio collection mutants were struck on LB + 25 µg/mL kanamycin from the cryogenic stock plate prepared during primary screening above and biological triplicate colonies were inoculated into a 96 well deepwell plate containing 300 µL M9 + 1% glucose. The next day, cells were inoculated into plates for cultivation on the Growth Profiler as described above.

Cross-compound tolerance screening

96 well deepwell plates containing 300 µL of M9 + 1% glucose were inoculated directly from cryogenic stocks made from precultures for the secondary screening of ALE isolates and were grown overnight at 37°C with 300 rpm shaking. The next day, cells were diluted 1:100 into 96 well half-deepwell plates containing the following final concentrations of each chemical in M9 + 1% glucose:

butanol	1.4% v/v
glutarate	40 g/L
p-coumarate	7.5 g/L
putrescine	32 g/L
HMDA	32 g/L
adipate	45 g/L
isobutyrate	7.5 g/L
hexanoate	3 g/L
octanoate	8 g/L
2,3-butanediol	6% v/v
1,2-propanediol	6% v/v
sodium chloride	0.6 M

Plates were cultivated in a Growth Profiler for 48 hours as described for screening of ALE isolates. Green pixel integrated values from each well were converted to OD_{600} values using a calibration curve and the resulting OD_{600} vs. elapsed time data was processed using custom scripts to determine the time required for each culture to reach an OD of 1.0 (t_{OD1}). This value is a combined measure of growth rate and lag time in each culture. The median value was taken for biological triplicates of each isolate and was normalized to the median t_{OD1} for K-12 MG1655 controls (5 replicates). The ratio of $t_{OD1(evolved)}/t_{OD1(wild\text{-}type)}$ is presented.

Results

Wild-type tolerance to diacids

The maximum measured concentration of glutaric acid at which exponentially growing K-12 MG1655 can grow was found to be 50 g/L with severe inhibition (Table 5). Increasing inhibition of growth was observed from 10 to 50 g/L.

Table 5. *Growth of K-12 MG1655 in varying concentrations of glutaric acid (neutralized with sodium hydroxide).*

	Mean		std. error	
glutaric acid (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.661	0.7	0.011	0.1
10	0.526	2.0	0.019	0.1
20	0.377	1.9	0.006	0.3
30	0.234	0.6	0.034	1.1
40	0.132	0.7	0.028	1.7
50	0.096	15.9	0.062	0.8
75	0.000	-	0.000	-

The maximum measured concentration of adipic acid at which exponentially growing K-12 MG1655 can grow was found to be 75 g/L with an extensive lag phase of 27 hours (Table 6). Growth rates dropped sharply as a function of concentration between 10 and 50 g/L.

Table 6. *Growth of K-12 MG1655 in varying concentrations of adipic acid (neutralized with sodium hydroxide).*

	Mean		std. error	
adipic acid (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.653	0.6	0.007	0.0
10	0.575	1.0	0.018	0.1
20	0.494	1.3	0.017	0.2

30	0.367	1.8	0.030	0.7
40	0.263	4.0	0.012	0.4
50	0.120	2.4	0.009	1.6
75	0.203	27.2	0.082	0.6
100	0.000	-	0.000	-

Growth was also tested in pimelic acid (C_7) and sebacic acid (C_{10}) (Table 7). Robust growth was still observed in pimelic acid at 45 g/L, however inhibition was observed as a function of increasing concentration. Sebacic acid was more toxic, with nearly no growth detected above 40 g/L concentration.

Table 7. *Growth of K-12 MG1655 in varying concentrations of pimelic and sebacic acids (neutralized with sodium hydroxide).*

	pimelic acid				sebacic acid			
	mean		std. error		Mean		std. error	
concentration (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.650	0.5	0.027	0.2	0.668	0.5	0.016	0.0
10	0.694	1.9	0.021	0.2	0.707	1.9	0.048	0.2
20	0.656	2.4	0.022	0.1	0.578	2.7	0.057	0.3
25	0.572	2.5	0.021	0.2	0.413	3.3	0.003	0.2
30	0.560	3.3	0.021	0.2	0.386	5.7	0.054	0.2
35	0.534	4.1	0.006	0.0	0.306	10.8	0.026	0.4
40	0.439	4.3	0.008	0.3	0.222	22.5	0.030	0.6
45	0.280	4.6	0.007	0.3	0.051	50.6	0.020	3.4

Aiming for a starting growth rate between 0.3 to 0.4 h^{-1}, it was decided to begin evolutions at a concentration of 20 g/L glutaric acid and 25 g/L adipic acid.

Resequencing of tolerant isolates

Variants detected in glutaric and adipic acid evolved strains are presented in Tables 8 and 9. Each strain name corresponds to the chemical the strain was isolated from, the population the strain was isolated from, and the original number of the strain assigned during primary screening (*e.g.* GLUT1-3 is a glutaric acid-evolved strain isolated from population 1). In each

table, strains are arranged such that all that were isolated from the same population are presented in the same rows. Strains with an asterisk (*) following their name are hypermutator strains, and only the mutation identified that can be associated with generating the hypermutator phenotype (here only in *mutS* or *mutT* in 1 isolate from 1 glutaric acid population and all isolates from 1 adipic acid population) and those mutations that are shared with other mutations in the same gene in other strains are shown.

Mutations that occur independently across multiple populations, or that appear fixed in a highly variable population are likely causative and of highest interest. For glutaric acid, these include mutations in *kgtP* or its promoter region (24 out of 24 isolates), *spoT* (all isolates except in population GLUT8), *rpoC* (9 isolates in 5 populations), *proV* (6 isolates in 3 populations) and *proX* (2 isolates in 1 population), *rnt* (5 isolates in 3 populations), *nagC* and *nagA* (4 isolates in 2 populations). In place of mutations in *spoT*, a coding mutation in *polB* was found in all 3 isolates of population GLUT8. Mutations in *rpoB*, encoding another subunit of RNA polymerase in addition to *rpoC*, were found in all 3 isolates from population GLUT5. Of these mutations, those of *kgtP*, *proV* and *proX*, and *nagC* are likely loss-of-function mutations, due to the presence of frameshift mutations, premature stop codons, or IS element insertions in at least one population of individual isolate that possesses mutations in that gene. Other mutations are likely gain-of-function or weakening of function, for example coding mutations in genes encoding subunits of RNA polymerase (RpoC and RpoB), SpoT (plus an in-frame deletion in one population), and PolB.

For adipic acid, mutated genes that occurred across multiple populations included those in *kgtP* (19 out of 19 isolates), *ybjL* (12 isolates in 6 populations), *proV* or its promoter region (11 isolates in 5 populations; plus 3 isolates in 1 population that possessed a large deletion spanning *proV*, *proX*, and *proW* plus other neighboring genes), *sspA* (7 isolates in 4 populations; also found in 1 isolate from glutaric acid), the intergenic region between *pyrE* and *rph* (6 isolates in 3 populations), *nagC* (5 isolates in 2 populations), *yicC* (5 isolates in 2 populations), *spoT* (4 isolates in 2 populations), and *pstS* or its promoter region (4 isolates in 2 populations). Notably lacking were mutations in any subunit of RNA polymerase. Of these mutations, those of *kgtP*, *ybjL*, *proV*, *sspA,* and *nagC* are likely loss-of-function mutations, due to the presence of frameshift mutations, premature stop codons, or IS element insertions in at least one population of individual isolate that possesses mutations in that gene. Coding mutations in SpoT are likely gain-of-function or weakening of function, as for glutaric acid. Mutations in *sspA* are either coding SNPs or an in-frame (21 bp) deletion, therefore it is unclear whether this mutation is a loss-of-function. PstS is one subunit of a transporter complex complex PstBACS which is involved in the import of inorganic phosphate under phosphate starvation conditions.

Table 8. *Variants detected in glutaric acid-evolved isolates*

coordinate	gene	change
GLUT1-3		
1347104	*rnb*	C603* (G→T)
1630841	*ydfI*	V25F (C→A)
2725668	*kgtP*	1 bp deletion
2726147	[*rrfG*][*rrsG*]	4703 bp deletion
3823664	*spoT*	V422A (T→C)
4186605	*rpoC*	H419P (A→C)
GLUT2-1		
481075	*tomB/acrB*	noncoding SNP (A→G)
2725642	*kgtP*	1 bp deletion
2804858	*proV*	13 bp deletion
3636414	*ygjP*	D152A (A→C)
3823751	*spoT*	A451D (C→A)
GLUT3-5		
2724971	*kgtP*	F259C (A→C)
3823770	*spoT*	W457C (G→T)
4186605	*rpoC*	H419P (A→C)
GLUT4-1		
2724611	*kgtP*	A379V (G→A)
2807193	*proX*	8 bp deletion
3824137	*spoT*	D580Y (G→T)

coordinate	gene	change
GLUT1-9		
1347104	*rnb*	C603* (G→T)
2725668	*kgtP*	1 bp deletion
2726129	[*rrfG*][*rrsG*]	4742 bp deletion
3823664	*spoT*	V422A (T→C)
4186605	*rpoC*	H419P (A→C)
GLUT2-9		
1347882	*rnb*	1 bp deletion
2725642	*kgtP*	1 bp deletion
3823751	*spoT*	A451D (C→A)
GLUT3-7		
2724971	*kgtP*	F259C (A→C)
3823770	*spoT*	W457C (G→T)
4186605	*rpoC*	H419P (A→C)
GLUT4-4		
2390019	*yfbP/nuoN*	G → A
2724611	*kgtP*	A379V (G→A)
2807193	*proX*	8 bp deletion
3195220	*ibsE/rfaE*	IS186 element insertion
3824137	*spoT*	D580Y (G→T)

coordinate	gene	change
GLUT1-10		
2725668	*kgtP*	1 bp deletion
2726161	[*rrfG*][*rrsG*]	4703 bp deletion
2805532	*proV*	1 bp insertion (→T)
3328463	*greA*	IS4 element insertion
3377214	*sspA*	21 bp amplification (X2)
3522182	*hofM*	noncoding SNP (C→A)
3751884	*yiaT/yiaU*	IS5 element insertion
3823664	*spoT*	V422A (T→C)
GLUT2-10		
1654069	*rspA*	T358S (G→C)
2725642	*kgtP*	1 bp deletion
2804858	*proV*	13 bp deletion
3823751	*spoT*	A451D (C→A)
4186605	*rpoC*	H419P (A→C)
GLUT3-9		
318484	*ykgI*	noncoding SNP (C→T)
996768	*ssuA*	noncoding SNP (G→A)
1728882	*rnt*	Q179P (A→C)
2725518	*kgtP*	1 bp insertion (→C)
3823759	*spoT*	N454H (A→C)
3969048	*wzzE*	1 bp insertion (→G)
GLUT4-10		
2724971	*kgtP*	F259C (A→C)
2788702	*ygaQ/csiD*	IS5 element insertion
3823751	*spoT*	A451V (C→T)
4186605	*rpoC*	H419P (A→C)

coordinate	gene	change
GLUT5-4		
303119	*yagU*	1 bp deletion
2725374	*kgtP*	1 bp deletion
3377359	*sspA*	18 bp deletion
3823106	*spoT*	R236L (G→T)
4181852	*rpoB*	K203T (A→C)
4451123	*ytfR*	noncoding SNP (C→A)
GLUT6-4		
657215	*pagP/cspE*	25 bp deletion
700680	*nagC*	IS1 element insertion
2725370	*kgtP*	L126* (A→C)
2765412	*yfjL/yfjM*	8 bp deletion
3823105	*spoT*	R236S (C→A)
GLUT7-2		
701396	*nagC*	Q67* (G→A)
1728926	*rnt*	F194L (T→C)
2724848	*kgtP*	G300V (C→A)
3824201	*spoT*	6 bp deletion
GLUT8-5		
64352	*polB*	R477G (G→C)
2725818	*kgtP/rfG*	noncoding SNP (A→G)
2804858	*proV*	13 bp deletion
2810987	*mprA*	IS1 element insertion
4185540	*rpoC*	P64L (C→T)
GLUT5-5		
2725374	*kgtP*	1 bp deletion
3377491	*sspA/rpsI*	IS2 element insertion
3823106	*spoT*	R236L (G→T)
4181852	*rpoB*	K203T (A→C)
4451123	*ytfR*	noncoding SNP (C→A)
GLUT6-5		
657215	*pagP/cspE*	25 bp deletion
700680	*nagC*	IS1 element insertion
2725370	*kgtP*	L126* (A→C)
2765412	*yfjL/yfjM*	8 bp deletion
3823105	*spoT*	R236S (C→A)
GLUT7-6		
1636300	*ydfJ*	T→G
1728926	*rnt*	F194L (T→C)
2724848	*kgtP*	G300V (C→A)
3824201	*spoT*	6 bp deletion
GLUT8-6		
64352	*polB*	R477G (G→C)
702331	*nagA*	1 bp insertion (→T)
1354284	*sapC*	G79W (C→A)
2725232	*kgtP*	G172V (C→A)
2804858	*proV*	13 bp deletion
4185540	*rpoC*	P64L (C→T)
GLUT5-9		
2725374	*kgtP*	1 bp deletion
3377491	*sspA/rpsI*	IS2 element insertion
3823106	*spoT*	R236L (G→T)
4181852	*rpoB*	K203T (A→C)
4451123	*ytfR*	noncoding SNP (C→A)
GLUT6-10		
1728425	*rnt*	A27T (G→A)
2672970	*hcaD*	6 bp deletion
2724725	*kgtP*	6 bp insertion (→CAAAAG)
3823139	*spoT*	M247K (T→A)
GLUT7-7*		
701377	*nagC*	1 bp deletion
1728884	*rnt*	A180T (G→A)
2725668	*kgtP*	1 bp deletion
2859432	*mutS*	noncoding SNP (C→T)
3823724	*spoT*	T442I (C→T)
GLUT8-9		
64352	*polB*	R477G (G→C)
1354284	*sapC*	G79W (C→A)
1907448	*yobF*	IS5 element insertion
2725208	*kgtP*	9 bp deletion
2804921	*proV*	1 bp deletion
2927703	*ygdH/sdaC*	intergenic SNP (G→A)
4185540	*rpoC*	P64L (C→T)

Table 9. *Variants detected in adipic acid-evolved isolates*

coordinate	gene	change	coordinate	gene	change	coordinate	gene	change
ADIP1-1			**ADIP1-9**					
814029	*uvrB*	D168E (T→G)	546309	*allD*	A19T (C→T)			
889562	*ybjL*	1 bp deletion	702405	*nagA*	36 bp amplification (X2)			
2725207	*kgtP*	IS1 element insertion	889562	*ybjL*	1 bp deletion			
2804648	*nrdF/proV*	38 bp deletion	2530235	*ligA/zipA*	noncoding SNP (G→T)			
3377068	*sspA*	21 bp deletion	2725207	*kgtP*	IS1 element insertion			
3815823	*pyrE/rph*	noncoding SNP (C→A)	2804648	*nrdF/proV*	38 bp deletion			
3816848	*yicC*	T58M (C→T)	3377068	*sspA*	21 bp deletion			
4294366	*nrfG/gltP*	noncoding SNP (A→T)	3815823	*pyrE/rph*	noncoding SNP (C→A)			
			3816848	*yicC*	T58M (C→T)			
			4294366	*nrfG/gltP*	noncoding SNP (A→T)			
ADIP2-5			**ADIP2-6**			**ADIP2-10**		
362830	*lacY*	C117F (C→A)	362830	*lacY*	C117F (C→A)	700628	*nagC*	IS5 element insertion
700529	*nagC*	IS1 element insertion	2725613	*kgtP*	S45L (G→A)	1530007	*ydcD*	S29* (C→A)
2725613	*kgtP*	S45L (G→A)	2798606	*alaE-ygaY*	10942 bp deletion	2725613	*kgtP*	S45L (G→A)
2798606	*alaE-ygaY*	10942 bp deletion	3815883	*rph*	2 bp deletion	2798606	*alaE-ygaY*	10942 bp deletion
3815883	*rph*	2 bp deletion				3815809	*pyrE/rph*	1 bp deletion
ADIP3-2			**ADIP3-4**			**ADIP3-8**		
889534	*ybjL*	IS5 element insertion	233954	*mltD*	2 bp insertion (→TG)	889534	*ybjL*	IS5 element insertion
2614996	*yfgO*	noncoding SNP (A→G)	889534	*ybjL*	IS5 element insertion	2725207	*kgtP*	IS1 element insertion
2725207	*kgtP*	IS1 element insertion	1879829	*yeaR*	IS186 element insertion	2804648	*nrdF/proV*	38 bp deletion
2804648	*nrdF/proV*	38 bp deletion	2725207	*kgtP*	IS1 element insertion	3377068	*sspA*	21 bp deletion
3377068	*sspA*	21 bp deletion	2804648	*nrdF/proV*	38 bp deletion	3815823	*pyrE/rph*	noncoding SNP (C→A)
3815823	*pyrE/rph*	noncoding SNP (C→A)	3377068	*sspA*	21 bp deletion	3816848	*yicC*	T58M (C→T)
3816848	*yicC*	T58M (C→T)	3633911	*yhiL*	IS5 element insertion			
			3815823	*pyrE/rph*	noncoding SNP (C→A)			
			3816848	*yicC*	T58M (C→T)			
ADIP4-8								
702405	*nagA*	36 bp amplification (X2)						
889488	*ybjL*	IS1 element insertion						
1728708	*rnt*	N121S (A→G)						
2675452	*yphC*	P104A (G→C)						
2693818	*purL*	V576L (C→G)						
2701175	*pdxJ*	noncoding SNP (G→C)						
2713302	*srmB*	R136L (G→T)						
2724590	*kgtP*	G386D (C→T)						
3548179	*malQ*	A631D (G→T)						
4130167	*metL*	462 bp deletion						

coordinate	gene	change	coordinate	gene	change	coordinate	gene	change
ADIP5-2*			**ADIP5-6***					
111305	*mutT*	E88* (G→T)	111305	*mutT*	E88* (G→T)			
888480	*ybjL*	F447C (A→C)	888948	*ybjL*	E291A (T→G)			
2724576	*kgtP*	Y391D (A→C)	2615642	*yfgO*	noncoding SNP (T→G)			
2805674	*proV*	L287* (T→G)	2724576	*kgtP*	Y391D (A→C)			
3549583	*malQ*	D163A (T→G)	2805674	*proV*	L287* (T→G)			
			3377387	*sspA*	T12P (T→G)			
ADIP6-3			**ADIP6-9**			**ADIP6-10**		
700928	*nagC*	G223* (C→A)	700928	*nagC*	G223* (C→A)	700928	*nagC*	G223* (C→A)
889534	*ybjL*	IS5 element insertion	889534	*ybjL*	IS5 element insertion	889534	*ybjL*	IS5 element insertion
1915297	*proQ*	R80C (G→A)	1196319	*icd*	noncoding SNP (C→A)	1915297	*proQ*	R80C (G→A)
2725155	*kgtP*	R198S (G→T)	1389396	*ycjG*	L156P (T→C)	2725155	*kgtP*	R198S (G→T)
2804858	*proV*	13 bp deletion	1915297	*proQ*	R80C (G→A)	2805493	*proV*	E227* (G→T)
3823700	*spoT*	S434L (C→T)	2725155	*kgtP*	R198S (G→T)	3823700	*spoT*	S434L (C→T)
4019173	*ubiE*	K107E (A→G)	2804831	*proV*	7 bp deletion			
			3823700	*spoT*	S434L (C→T)			
ADIP7-2			**ADIP7-5**					
293574	*yagL*	noncoding SNP (A→G)	889540	*ybjL*	IS5 element insertion			
1293038	*hns/tdk*	IS1 element insertion	2725642	*kgtP*	1 bp deletion			
1598223	*yneO/lsrK*	IS5 element insertion	2804858	*proV*	13 bp deletion			
2725329	*kgtP*	G140* (C→A)	3377240	*sspA*	T61P (T→G)			
2867354	*rpoS*	9 bp deletion	4490689	*idnR*	IS1 element insertion			
3910996	*pstS*	4 bp insertion (→CTTT)						
ADIP8-3			**ADIP8-7**			**ADIP8-10**		
1293015	*hns/tdk*	IS1 element insertion	1293015	*hns/tdk*	IS1 element insertion	280003	*insI1*	IS5 element insertion
2192447	*yehD/yehE*	1 bp deletion	2192447	*yehD/yehE*	1 bp deletion	2192447	*yehD/yehE*	1 bp deletion
2724588	*kgtP*	G387S (C→T)	2724588	*kgtP*	G387S (C→T)	2724588	*kgtP*	G387S (C→T)
3911563	*pstS/glmS*	IS1 element insertion	3911563	*pstS/glmS*	IS1 element insertion	3911563	*pstS/glmS*	IS1 element insertion

Characterization of selected isolates

Each re-sequenced isolate was characterized using the Biolector system for growth at the screening concentration of chemical (47.5 g/L glutaric acid or 50 g/L adipic acid) in biological triplicates. The average growth rates with standard errors for the three replicates are shown in Tables 10 and 11.

Variations in growth behavior amongst evolved isolates can be noted. Better growing strains are defined by both the slope of the curve (higher growth rate) and at what time the cultures begin growing (reduced lag time). Some isolates exhibit poorer improvements in growth rates (*e.g.* ADIP7-2 and ADIP8 isolates) but especially reduced lag times. The phenotype to genotype relationship infers mutations that are of highest interest and those that are not of interest. For example, GLUT2-10 was the best performing isolate from population GLUT2, indicating that either the RpoC-H419P and/or RpsA-T358S mutations are causative for higher growth rate, or the lack of other mutations found in the other two isolates is beneficial. Another example of this would, for example, be when multiple isolates from one population are growing nearly identically (*e.g.*GLUT4-1 and GLUT4-4). This indicates that any differences in mutations between these two isolates are not important for tolerance, in this case the intergenic mutations between *yfbP* and *nuoN*, and between *ibsE* and *rfaE,* found in GLUT4-4.

Table 10. *Growth rates and lag times of re-sequenced glutaric acid evolved isolates in M9 + 47.5 g/L glutaric acid (neutralized with sodium hydroxide).*

	mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.103	10.7	0.020	4.7
GLUT1-3	0.304	5.1	0.012	1.8
GLUT1-9	0.319	6.4	0.012	0.7
GLUT1-10	0.458	11.3	0.085	0.1
GLUT2-1	0.276	6.8	0.077	0.6
GLUT2-9	0.298	6.5	0.014	1.5
GLUT2-10	0.378	6.5	0.051	0.5
GLUT3-5	0.284	5.6	0.021	2.1
GLUT3-7	0.277	5.6	0.044	3.1

GLUT3-9	0.324	8.3	0.014	3.8
GLUT4-1	0.279	6.2	0.007	1.1
GLUT4-4	0.287	6.6	0.015	1.1
GLUT4-10	0.297	6.9	0.020	2.8
GLUT5-4	0.438	17.0	0.098	18.0
GLUT5-5	0.349	14.5	0.054	13.8
GLUT6-4	0.277	5.9	0.013	0.7
GLUT6-5	0.300	6.5	0.025	0.7
GLUT6-10	0.341	6.7	0.093	0.9
GLUT7-2	0.318	6.6	0.027	0.2
GLUT7-6	0.318	7.1	0.010	1.2
GLUT7-7	0.283	7.8	0.023	0.8
GLUT8-5	0.322	6.2	0.018	0.9
GLUT8-6	0.347	7.1	0.013	0.9
GLUT8-9	0.345	7.4	0.042	2.4

Table 11. *Growth rates and lag times of re-sequenced adipic acid evolved isolates in M9 + 50 g/L adipic acid (neutralized with sodium hydroxide).*

	mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655-1	0.133	21.4	0.017	2.1
ADIP1-1	0.315	16.6	0.037	5.6
ADIP1-9	0.274	5.8	0.013	1.4
ADIP2-5	0.332	20.1	0.019	2.4
ADIP2-6	0.337	22.1	0.038	2.0
ADIP2-10	0.326	20.8	0.007	1.6
ADIP3-2	0.295	16.6	0.011	4.1
ADIP3-4	0.305	23.3	0.017	11.5

ADIP3-8	0.295	20.1	0.032	4.7
ADIP4-8	0.332	6.8	0.022	0.7
ADIP5-2	0.401	4.7	0.015	1.3
ADIP5-6	0.370	14.4	0.130	7.9
ADIP6-3	0.280	6.5	0.003	0.4
ADIP6-9	0.279	6.8	0.014	0.5
ADIP6-10	0.284	7.1	0.023	0.2
ADIP7-2	0.189	7.6	0.024	4.4
ADIP7-5	0.292	19.3	0.006	12.7
ADIP8-3	0.201	6.0	0.011	1.4
ADIP8-7	0.206	7.2	0.011	0.5
ADIP8-10	0.200	6.8	0.016	1.9

Sole carbon source plate growth assay

Wild-type, glutaric acid, and adipic acid evolved strains were struck on M9 agar containing glutarate or adipate as a sole carbon source. No growth was observed on adipic acid plates, indicating that *E. coli* cannot utilize adipic acid as a sole carbon source. Robust but very slow growth of wild-type K-12 MG1655 was observed after a few weeks (Table 12), indicating the ability of *E. coli* to use glutarate as a sole carbon source, almost certainly through promiscuous activity of pathway enzymes (because glutarate is not a natural metabolite in *E. coli*). Growth on this compound as a sole carbon source has not been previously reported in the literature. Evolved isolates generally could not grow on glutaric acid, with the exception of weak growth exhibited by GLUT8-9. The only common genetic feature between all evolved isolates relative to the wild-type are probable loss-of-function mutations in *kgtP*, implicating KgtP, an α-ketoglutarate importer, as being a direct importer for glutarate. GLUT8-9 notably features an in-frame 9 bp deletion in *kgtP* that is unique among all the isolates. Without being limited to theory, this mutation may result in a reduced activity of KgtP, rather than a full loss-of-function.

Table 12. *Qualitative growth score of glutarate-evolved isolates on M9 agar plates containing 10 g/L glutaric acid (neutralized) as a sole carbon source.*

	growth score
MG1655	+++
GLUT1-3	None
GLUT1-9	None
GLUT1-10	None
GLUT2-1	None
GLUT2-9	None
GLUT2-10	None
GLUT3-5	None
GLUT3-7	None
GLUT3-9	None
GLUT4-1	None
GLUT4-4	None
GLUT4-10	None
GLUT5-4	None
GLUT5-5	None
GLUT6-4	None
GLUT6-5	None
GLUT6-10	None
GLUT7-2	None
GLUT7-6	None
GLUT7-7	None
GLUT8-5	None
GLUT8-6	None
GLUT8-9	+

Knockout strain growth performance

High glutaric acid concentrations:

Probable loss-of-function mutations were identified from re-sequencing results as described in methods and in the results of the resequencing analysis. Because there was probable loss-of-function of *kgtP* in all resequenced isolates, this was the only single knockout tested, and additional knockouts were selected to be tested as double combinations together with *kgtP* (Table 13). Only the triple knockout in *kgtP*, *proV*, and *nagC* was tested initially due to one isolate (GLUT8-6) possessing probable loss-of-function mutations in *kgtP*, *proV*, and *nagA*, and due to previous studies indicating similar phenotypes and likely the same mechanism of action for improved tolerance in high osmotic pressures (or due to high Na^+ concentrations) from both *nagC* and *nagA* knockouts (Lennen and Herrgård, 2014). All strains with *kgtP* knockouts exhibited higher growth rates than the wild-type in 23.8 g/L and 47.5 g/L glutaric acid, however none of the multiple knockout strains exhibited significantly improved growth relative to the single *kgtP* knockout strain alone. K-12 MG1655 Δ*kgtP nagC::kan* exhibited reduced growth relative to K-12 MG1655 *kgtP::kan* in 47.5 g/L glutaric acid.

Table 13. *Growth rates and lag times of preliminary selections of single, double, and triple gene knockout mutants in M9 + 23.8 g/L or 47.5 g/L glutaric acid (neutralized), as measured in the Growth Profiler testing format.*

	23.8 g/L glutarate				47.5 g/L glutarate			
	mean		std. error		mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.466	7.4	0.010	0.1	0.101	21.8	0.032	0.7
MG1655 kgtP::kan	0.487	6.1	0.019	0.0	0.191	13.5	0.019	0.4
MG1655 ΔkgtP proV::kan	0.520	6.2	0.019	0.1	0.251	13.0	0.019	0.2
MG1655 ΔkgtP nagC::kan	0.491	6.7	0.019	0.1	0.179	15.0	0.007	0.5
MG1655 ΔkgtP rnb::kan	0.505	6.3	0.012	0.0	0.183	13.6	0.017	0.8
MG1655 ΔkgtP sspA::kan	0.473	6.1	0.007	0.1	0.225	12.2	0.008	0.4
MG1655 ΔkgtP ΔproV nagC::kan	0.541	6.6	0.006	0.2	0.215	14.0	0.016	0.9

A second group of selected single, double, and triple knockout mutants was tested in the Biolector testing format in M9 + 47.5 g/L glutaric acid (Table 14). The *ybjL* loss-of-function which had been identified from resequencing of adipic acid evolved isolates was included, to determine if that mutation would also confer tolerance toward glutaric acid. The *proV* and *ybjL* mutations did not increase growth rates alone, but in double combinations with the *kgtP* mutation, were found to increase the growth rate over that of the *kgtP* single mutant. K-12

MG1655 Δ*kgtP sspA::kan* additionally exhibited an increased growth rate over that of the *kgtP* single knockout mutant. The triple knockout mutant in *kgtP*, *proV*, and *ybjL* exhibited a growth rate higher than that of the tested double knockout combinations, with a growth rate nearly equivalent to two of the evolved isolates tested alongside in the same experiment (GLUT1-3 and GLUT4-1), and exceeding the growth rate of many other evolved isolates in Table 6.

Table 14. *Growth rates and lag times of selected single, double, and triple gene knockout mutants in M9 + 47.5 g/L glutaric acid (neutralized), as measured in the Biolector testing format.*

	mean		std. error	
Strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.100	10.4	0.018	2.8
GLUT1-3	0.278	4.6	0.014	0.6
GLUT1-10	0.442	10.8	0.009	0.1
GLUT4-1	0.312	7.1	0.009	0.1
GLUT8-6	0.402	7.8	0.001	0.2
MG1655 kgtP::kan	0.181	5.8	0.005	0.6
MG1655 proV::kan	0.085	0.6	0.008	2.4
MG1655 ybjL::kan	0.126	12.8	0.006	0.7
MG1655 ΔkgtP proV::kan	0.244	6.5	0.000	0.2
MG1655 ΔkgtP sspA::kan	0.256	5.7	0.007	0.9
MG1655 ΔkgtP ybjL::kan	0.213	6.0	0.008	0.3
MG1655 ΔkgtP ΔproV ybjL::kan	0.300	7.0	0.013	0.2

<u>High adipic acid concentrations</u>

Probable loss-of-function mutations were identified as previously described. As for glutarate, probable loss-of-function of *kgtP* was identified in all resequenced isolates, therefore this single knockout was tested with additional double and triple combinations all containing the *kgtP* knockout. Of the tested strains, K-12 MG1655 *kgtP::kan* exhibited slightly improved tolerance in 25 g/L adipate, and a much larger improvement in growth in 50 g/L adipate

(Table 15). The only tested combinatorial knockout with a higher growth rate than the *kgtP* single knockout strain was MG1655 Δ*kgtP proV::kan*. K-12 MG1655 Δ*kgtP sspA::kan* exhibited greatly reduced tolerance relative to the wild-type, indicating that the *sspA* mutations isolated in resequenced mutants are likely either gain-of-function mutations or that they only result in weakened activity of the gene product.

Table 15. *Growth rates and lag times of preliminary selections of single, double, and triple gene knockout mutants in M9 + 25 g/L or 50 g/L adipic acid (neutralized), as measured in the Growth Profiler testing format.*

	25 g/L adipate				50 g/L adipate			
	mean		std. error		mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.467	6.9	0.015	0.1	0.115	25.7	0.004	0.7
MG1655 kgtP::kan	0.534	6.4	0.003	0.2	0.192	17.5	0.002	0.6
MG1655 ΔkgtP proV::kan	0.583	6.2	0.029	0.2	0.264	16.5	0.005	0.2
MG1655 ΔkgtP pstS::kan	0.465	7.6	0.006	0.2	0.139	21.3	0.005	0.3
MG1655 ΔkgtP sspA::kan	0.525	6.8	0.006	0.4	0.117	49.2	0.029	12.4
MG1655 ΔkgtP ΔproV nagC::kan	0.501	6.8	0.008	0.2	0.213	19.7	0.009	0.7

In a second experiment, additional single, double, and triple knockout combinations were tested in 25 g/L and 50 g/L adipate (Table 16).

Table 16. *Growth rates and lag times of additional selections of single, double, and triple gene knockout mutants in M9 + 25 g/L or 50 g/L adipic acid (neutralized), as measured in the Growth Profiler testing format.*

	25 g/L adipate				50 g/L adipate			
	mean		std. error		mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.558	7.5	0.030	0.1	0.080	52.5	0.024	2.8
MG1655 kgtP::kan	0.575	6.3	0.017	0.2	0.189	16.0	0.002	0.6
MG1655 ΔkgtP proV::kan	0.608	6.2	0.032	0.2	0.255	15.1	0.010	0.7
MG1655 ybjL::kan	0.516	7.4	0.020	0.1	0.142	26.1	0.010	1.0
MG1655 ΔkgtP ybjL::kan	0.552	6.3	0.029	0.1	0.254	13.5	0.006	0.5
MG1655 ΔkgtP ΔproV ybjL::kan	0.586	6.3	0.011	0.2	0.326	12.6	0.019	0.7

The *ybjL* single knockout strain was found to moderately improve tolerance, but below the levels conferred by deletion of *kgtP.* K-12 MG1655 Δ*kgtP ybjL::kan* exhibited growth rates similar to K-12 MG1655 Δ*kgtP proV::kan*, and the triple knockout combination found in K-12 MG1655 Δ*kgtP* Δ*proV ybjL::kan* exhibited an improved growth rate over either double knockout combination. Selected single, double, and triple knockout strains were then tested in the Biolector testing format (Table 17). Results were similar to that observed in Table 16, with a higher growth rate observed for K-12 MG1655 Δ*kgtP* Δ*proV ybjL::kan* than for some ALE isolates. K-12 MG1655 Δ*kgtP sspA::kan* was additionally tested, however growth rates were not improved for media containing adipate as they were for glutarate.

Table 17. *Growth rates and lag times of selected single, double, and triple gene knockout mutants in M9 + 50 g/L adipic acid (neutralized), as measured in the Biolector testing format.*

	mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.188	13.8	0.030	1.4
ADIP1-9	0.284	8.1	0.011	9.0
ADIP4-8	0.351	6.1	0.006	0.2
ADIP6-3	0.311	6.3	0.008	0.2
ADIP8-3	0.208	4.5	0.004	2.7
MG1655 kgtP::kan	0.225	11.5	0.004	0.4
MG1655 proV::kan	0.153	19.2	0.003	0.5
MG1655 ybjL::kan	0.195	10.9	0.005	0.0
MG1655 ΔkgtP proV::kan	0.262	11.0	0.007	0.1
MG1655 ΔkgtP ybjL::kan	0.229	10.4	0.007	0.7
MG1655 ΔkgtP sspA::kan	0.164	20.5	0.056	10.0
MG1655 ΔkgtP ΔproV ybjL::kan	0.298	9.7	0.034	2.2

Screening of a single deletion mutant collection for diacid tolerance

To determine if any additional single gene deletion candidates were overlooked, screening on elevated glutarate and adipate concentrations was also conducted using the Keio collection of

gene knockouts, which is a commercial collection of knockouts in nearly all non-essential genes and ORFs in *E. coli* strain BW25113. This strain is a K-12 derivative and possesses known mutations relative to the K-12 MG1655 background. All Keio collection strains with knockouts in genes that were found to be mutated in Tables 8 and 9 were screened for growth against the BW25113 control in M9 + 40 g/L or 47.5 g/L glutaric acid, or M9 + 45 g/L or 50 g/L adipic acid (neutralized with sodium hydroxide) in the Growth Profiler screening format. Primary screening hits were measured again in a secondary screen in biological replicates, with averaged growth curves for 3 biological replicate cultures shown individually for each strain in Tables 18 and 19. For glutaric acid (Table 18), BW25113 *cspE::kan* and BW25113 *proX::kan* exhibited the largest increases in growth rate at 47.5 g/L glutarate, with small improvements also seen with 40 g/L glutarate. ProX is a subunit with ProV in the ProVWX ABC transporter. In M9 + 47.5 g/L glutarate, additional knockout strains with smaller improvements in growth rates were the *rfaE, yfbP*, and *yfjM* knockout strains.

For adipic acid (Table 19), a number of single deletion mutants exhibited moderate increases in growth rate in 50 g/L adipate. These were knockouts in *proQ*, *pstS*, *rph*, *rpoS*, *sspA*, *tdk*, *uvrB*, *ycjG*, and *yeaR*. In 47.5 g/L adipate, smaller percentage improvements in growth rate were observed, however all of these mutants similar exhibited significant increases in growth rate.

Table 18. *Growth rates of Keio collection knockouts in M9 + 40 g/L and 47.5 g/L glutaric acid (neutralized with sodium hydroxide) as measured in the Growth Profiler testing format.*

	40 g/L glutarate		47.5 g/L glutarate	
Strain	μ (h^{-1})	std. error	μ (h^{-1})	std. error
BW25113	0.227	0.010	0.090	0.011
BW25113 kgtP::kan	0.346	-	0.278	-
BW25113 cspE::kan	0.248	0.012	0.138	0.008
BW25113 greA::kan	0.228	0.017	0.094	0.003
BW25113 mprA::kan	0.219	0.017	0.096	0.015
BW25113 polB::kan	0.205	0.012	0.097	0.005
BW25113 proX::kan	0.261	0.005	0.140	0.011
BW25113 rfaE::kan	0.220	0.006	0.121	0.007
BW25113 ssuA::kan	0.207	0.007	0.093	0.005

BW25113 yfbP::kan	0.216	0.009	0.118	0.009
BW25113 yfjM::kan	0.228	0.006	0.118	0.004
BW25113 ygdH::kan	0.215	0.014	0.098	0.007
BW25113 yiaU::kan	0.235	0.005	0.111	0.009

Table 19. *Growth rates of Keio collection knockouts in M9 + 45 g/L and 50 g/L adipic acid (neutralized with sodium hydroxide) as measured in the Growth Profiler testing format.*

	45 g/L adipate		50 g/L adipate	
strain	μ (h^{-1})	std. error	μ (h^{-1})	std. error
BW25113	0.158	0.009	0.072	0.010
BW25113 kgtP::kan	0.230	-	0.218	-
BW25113 idnR::kan	0.165	0.014	0.070	0.001
BW25113 lsrK::kan	0.154	0.006	0.070	0.017
BW25113 malQ::kan	0.162	0.017	0.077	0.002
BW25113 metL::kan	0.167	0.003	0.067	0.036
BW25113 nrfG::kan	0.174	0.007	0.064	0.005
BW25113 proQ::kan	0.189	0.016	0.096	0.006
BW25113 pstS::kan	0.201	0.011	0.107	0.011
BW25113 rph::kan	0.206	0.005	0.119	0.012
BW25113 rpoS::kan	0.189	0.002	0.113	0.001
BW25113 sspA::kan	0.186	0.003	0.116	0.021
BW25113 tdk::kan	0.205	0.006	0.113	0.003
BW25113 uvrB::kan	0.197	0.016	0.113	0.019
BW25113 ycjG::kan	0.195	0.015	0.127	0.019
BW25113 yeaR::kan	0.197	0.011	0.101	0.023

A list of all gene disruption mutants in both the K-12 MG1655 and BW25113 background strains that exhibited increased tolerance to glutaric acid is shown in Table 20. A similar table for adipic acid is shown in Table 21.

Table 20: *Summary of knockout strains with improved growth over the wild-type strain in glutaric acid*

Strain genotype	Growth rate improvement	Lag time improvement
K-12 MG1655 *kgtP::kan*	Moderate	large
K-12 MG1655 *ybjL::kan*	Small	none
K-12 MG1655 Δ*kgtP proV::kan*	Moderate	large
K-12 MG1655 Δ*kgtP ybjL::kan*	Moderate	large
K-12 MG1655 Δ*kgtP sspA::kan*	Moderate	large
K-12 MG1655 Δ*kgtP* Δ*proV ybjL::kan*	Large	large
BW25113 *kgtP::kan*	Large (40 and 47.5 g/L)	not quantified
BW25113 *cspE::kan*	small (40 and 47.5 g/L)	not quantified
BW25113 *proX::kan*	small (40 and 47.5 g/L)	not quantified
BW25113 *rfaE::kan*	minor (47.5 g/L)	not quantified
BW25113 *yfbP::kan*	minor (47.5 g/L)	not quantified
BW25113 *yfjM::kan*	minor (47.5 g/L)	not quantified

Table 21: *Summary of knockout strains with improved growth over the wild-type strain in adipic acid*

Strain genotype	Growth rate improvement	Lag time improvement
K-12 MG1655 *kgtP::kan*	Moderate	large
K-12 MG1655 *ybjL::kan*	Small	none
K-12 MG1655 Δ*kgtP proV::kan*	Moderate	large
K-12 MG1655 Δ*kgtP ybjL::kan*	Moderate	large

K-12 MG1655 Δ*kgtP* Δ*proV ybjL::kan*	Large	large
BW25113 *kgtP::kan*	Large (45 and 50 g/L)	not quantified
BW25113 *proQ::kan*	small (45 and 50 g/L)	not quantified
BW25113 *pstS::kan*	moderate (45 and 50 g/L)	not quantified
BW25113 *rph::kan*	moderate (45 and 50 g/L)	not quantified
BW25113 *rpoS::kan*	small (45 and 50 g/L)	not quantified
BW25113 *sspA::kan*	small (45 and 50 g/L)	not quantified
BW25113 *tdk::kan*	small (45 and 50 g/L)	not quantified
BW25113 *uvrB::kan*	small (45 and 50 g/L)	not quantified
BW25113 *ycjG::kan*	small (45 g/L), moderate (50 g/L)	not quantified
BW25113 *yeaR::kan*	small (45 and 50 g/L)	not quantified

Sole carbon source plate growth assay of knockout strains

The single knockout strains in *kgtP*, *proV*, and *ybjL*, plus the double and triple combination knockout strains, were struck as previously described on glutarate as a sole carbon source, together with wild-type K-12 MG1655 and a selection of ALE evolved isolates as controls. Robust growth was again observed from K-12 MG1655 after a few weeks incubation, with reduced growth of GLUT8-9 and greatly reduced or no growth in other evolved isolates (Table 22). A larger inoculum was spread on the plates, which likely explains why very weak growth was observed for GLUT8-6 and GLUT1-10. K-12 MG1655 *kgtP::kan* exhibited no growth, indicating that loss-of-function of *kgtP* is explicitly responsible for the weak or absent growth of evolved strains on glutarate, and suggesting that KgtP is indeed a direct importer of glutrate. The *proV* and *ybjL* single knockout strains did not exhibit reduced growth relative to K-12 MG1655, and double and triple knockout combination strains with *kgtP* did not exhibit any growth, as would be expected from the loss of *kgtP*.

Table 22. *Qualitative growth scores of selected controls (K-12 MG1655 and glutarate evolved isolates) plus selected single, double, and triple knockout mutants isolates on M9 agar plates containing 10 g/L glutaric acid (neutralized) as a sole carbon source.*

strain	growth score
MG1655	+++
GLUT1-3	none
GLUT1-10	+
GLUT4-1	none
GLUT8-6	+
GLUT8-9	+
MG1655 kgtP::kan	none
MG1655 proV::kan	+++
MG1655 ybjL::kan	+++
MG1655 ΔkgtP proV::kan	none
MG1655 ΔkgtP ybjL::kan	none
MG1655 ΔkgtP ΔproV ybjL::kan	none

Cross-compound tolerance testing

Every secondary screened evolved isolate from the glutaric acid and adipic acid evolutions was grown in the presence of every other compound in the study as indicated in the Methods. The normalized $t_{OD1(evolved\ strain)}/t_{OD1(wild\text{-}type)}$ are shown in Tables 23 and 24 for the glutaric acid and adipic acid evolved isolates, respectively. Lower values are indicative a larger improvement in growth of the evolved isolate (left column) in that chemical condition (top row), whereas higher values are indicative of a lower improvement or decrease in growth compared to the wild-type. Averaged ratios across conditions and strains shown at the right and bottom of the plot allow for overall by-chemical and by-strain trends to be observed. Strain names that are followed by an asterisk (*) were not re-sequenced, and strain names in italics were found to be hypermutator strains.

The majority of glutaric acid-evolved isolates exhibit cross-tolerance to adipic acid (notable exceptions were isolates from the GLUT5 population, GLUT1-3, GLUT1-9, and GLUT2-10). Likewise, the majority of adipic acid evolved isolates exhibit cross-tolerance to glutaric acid

(notable exceptions were most isolates in the ADIP1, ADIP2, and ADIP3 populations, plus a couple isolates from other populations (ADIP4-4, ADIP5-5, neither of which were resequenced). Isolates with the highest degree of cross-tolerance were GLUT4-10, GLUT8-6, and GLUT8-9. The GLUT8 population was notable for possessing coding mutations in *polB* and lacking coding mutations in *spoT*. Adipic acid evolved isolates exhibited a lower overall degree of cross tolerance, with the best performing isolate being ADIP6-9. This isolate most likely has loss-of-function of *kgtP*, *proV*, and *ybjL*, plus coding mutations in *proQ* (suggested to be loss-of-function from Keio mutant screen) and *spoT*. The ADIP6 population specifically exhibited a high level of cross-tolerance toward all other acid salts in the study (hexanoate, octanoate, isobutyrate, glutarate, and p-coumarate). Acid cross-tolerance was also evident from many glutaric acid evolved isolates, however high cross-tolerance toward the diamine HMDA and the diols 2,3-butanediol and 1,2-propanediol was evident in a number of isolates. Cross-tolerance toward HMDA in population GLUT8 can be inferred to be due to a mutation present in GLUT8-6 and GLUT8-9 that is not found in GLUT8-5, which in this case is a coding mutation in *sapC* that is likely causative for HMDA cross-tolerance.

Table 23. *Normalized $t_{OD1(evolved)}/t_{OD1(wild\text{-}type)}$ values for glutaric acid-evolved isolates grown in the presence of inhibitory concentrations of 12 different chemicals.*

	Butanol	glutarate	coumarate	2,3-butanediol	putrescine	HMDA	adipate	isobutyrate	hexanoate	octanoate	1,2-propanediol	NaCl	average
GLUT1-3	1.31	1.94	2.24	3.06	4.02	1.00	1.97	1.58	0.78	0.86	2.03	3.16	2.00
GLUT1-9	1.31	1.74	2.24	3.06	4.02	1.00	1.75	1.39	0.92	0.88	1.97	3.16	1.96
GLUT1-10	1.31	0.72	1.45	0.65	1.42	1.00	0.58	2.92	0.68	0.79	0.78	1.39	1.14
GLUT2-1	1.12	0.68	1.47	1.32	1.10	0.99	0.63	1.61	0.86	0.92	1.00	1.54	1.10
GLUT2-9	1.22	0.68	1.27	1.24	0.96	1.00	0.63	1.47	0.84	0.91	0.94	1.08	1.02
GLUT2-10	1.31	1.42	2.24	3.06	4.02	1.00	1.40	1.29	0.86	0.86	1.75	3.16	1.87
GLUT3-5	*1.31*	*0.58*	*1.05*	*0.98*	*0.90*	*1.00*	*0.53*	*1.21*	*0.70*	*0.52*	*0.81*	*2.28*	*0.99*
GLUT3-7	1.31	0.58	0.97	1.05	0.98	0.96	0.53	1.34	0.81	0.59	0.84	1.28	0.94
GLUT3-9	1.24	0.68	1.55	0.76	1.06	1.00	0.85	1.63	0.73	0.77	0.97	2.80	1.17
GLUT4-1	1.31	0.76	1.43	3.06	1.15	0.53	0.68	1.34	1.14	1.34	1.41	0.92	1.26
GLUT4-4	1.31	0.76	1.63	3.06	1.15	0.52	0.73	1.32	1.11	1.51	1.44	1.00	1.29
GLUT4-10	1.31	0.56	0.79	0.76	0.90	0.73	0.52	1.37	0.70	0.45	0.84	1.07	0.83

GLUT5-4	1.31	2.82	1.17	3.06	4.02	1.00	2.48	5.08	0.81	0.96	1.56	3.16	2.29
GLUT5-5	1.31	2.48	0.87	3.06	4.02	1.00	1.88	2.82	0.73	0.64	1.28	3.16	1.94
GLUT5-9	1.31	2.78	1.26	3.06	4.02	1.00	2.47	5.08	0.76	0.41	1.47	3.16	2.23
GLUT6-4	1.16	0.70	1.01	1.14	1.17	1.00	0.67	1.37	0.86	0.81	0.94	1.70	1.04
GLUT6-5	1.27	0.74	1.05	1.38	1.33	0.90	0.67	1.39	0.95	0.83	1.06	1.82	1.12
GLUT6-10	1.25	0.68	0.84	1.51	1.00	1.00	0.63	1.29	0.97	0.81	1.06	0.98	1.00
GLUT7-2	1.22	0.64	0.64	1.48	0.88	0.60	0.57	1.21	0.84	0.79	1.00	0.85	0.89
GLUT7-6	1.20	0.62	0.77	1.03	0.96	0.39	0.65	1.29	0.92	0.70	1.09	1.00	0.88
GLUT7-7	1.16	0.62	1.12	0.83	1.04	0.75	0.58	1.71	0.68	0.55	0.88	1.13	0.92
GLUT8-5	1.31	0.94	1.00	1.03	2.29	1.00	0.83	0.92	0.70	0.80	0.94	3.05	1.23
GLUT8-6	1.31	0.60	1.00	1.21	0.90	0.33	0.53	1.18	0.70	0.48	0.84	0.90	0.83
GLUT8-9	1.31	0.60	0.91	0.94	0.96	0.34	0.52	1.03	0.68	0.48	0.88	0.95	0.80
average	1.27	1.06	1.25	1.74	1.84	0.83	0.97	1.79	0.82	0.78	1.16	1.86	1.28
# >wt	0	18	7	6	8	11	18	1	22	22	11	5	
% >wt	0.0	75.0	29.2	25.0	33.3	45.8	75.0	4.2	91.7	91.7	45.8	20.8	

Table 24. *Normalized $t_{OD1(evolved)}/t_{OD1(wild\text{-}type)}$ values for adipic acid-evolved isolates grown in the presence of inhibitory concentrations of 12 different chemicals.*

	butanol	glutarate	coumarate	2,3-butanediol	putrescine	HMDA	Adipate	isobutyrate	hexanoate	octanoate	1,2-propanediol	NaCl	average
ADIP1-1	1.22	1.38	1.75	0.59	3.22	3.51	1.37	4.20	0.74	1.38	5.68	2.64	2.31
ADIP1-6*	1.25	0.96	1.75	0.59	3.22	3.51	0.73	3.13	1.00	1.24	0.74	2.64	1.73
ADIP1-9	1.27	1.32	1.75	0.58	3.22	3.51	1.34	4.20	0.81	1.38	0.79	2.64	1.90
ADIP2-5	1.17	1.09	1.06	0.87	2.77	3.51	1.28	0.87	0.67	1.38	5.68	2.64	1.92
ADIP2-6	1.22	0.95	1.09	0.71	2.48	3.51	0.96	0.87	0.63	1.38	0.79	2.64	1.44
ADIP2-10	1.18	1.32	0.99	1.10	2.87	3.51	1.16	0.91	0.65	1.38	3.26	2.64	1.75
ADIP3-2	*1.27*	*0.90*	*1.42*	*0.51*	*3.22*	*3.51*	*0.94*	*3.67*	*0.74*	*1.38*	*0.68*	*2.64*	*1.74*

ADIP3-4	1.27	1.35	1.75	0.59	3.22	3.51	1.16	4.20	0.84	1.38	1.24	2.64	1.93
ADIP3-8	1.18	0.85	1.35	0.50	3.22	3.51	1.37	3.91	0.74	1.38	0.85	2.64	1.79
ADIP4-1	1.27	0.50	1.15	0.73	0.93	1.00	0.47	1.30	0.84	0.94	1.15	0.95	0.94
ADIP4-4*	1.27	0.96	0.83	1.01	1.02	1.07	0.71	1.28	1.05	1.03	1.79	2.64	1.22
ADIP4-8	1.26	0.53	0.76	0.83	1.00	1.11	1.80	1.33	0.88	0.78	1.03	0.86	1.01
ADIP5-2	1.12	0.41	0.96	1.87	0.75	0.73	0.32	1.13	0.91	0.91	5.29	2.64	1.42
ADIP5-5*	1.27	1.06	0.98	1.87	1.02	1.11	0.93	1.15	1.26	0.89	1.18	1.22	1.16
ADIP5-6	1.18	1.37	0.77	0.77	3.22	3.51	1.17	2.07	0.88	0.81	1.97	2.64	1.70
ADIP6-3	1.16	0.42	0.55	1.87	0.90	1.27	0.40	1.91	0.70	0.53	1.12	0.79	0.97
ADIP6-9	1.09	0.45	0.55	0.62	0.88	1.11	0.38	1.30	0.70	0.56	0.91	0.84	0.78
ADIP6-10	1.12	0.58	0.89	0.68	1.02	1.11	0.47	3.35	0.84	0.89	1.47	1.42	1.15
ADIP7-2	1.27	0.53	0.63	0.73	1.22	1.55	0.47	0.91	0.84	1.38	1.00	1.07	0.96
ADIP7-5	1.27	0.68	1.13	0.59	1.92	1.93	0.56	3.93	0.77	1.06	1.29	2.47	1.47
ADIP7-10*	1.27	0.54	0.89	0.46	2.12	2.13	0.53	3.50	0.72	0.99	0.71	1.81	1.30
ADIP8-3	1.27	0.63	0.55	1.10	1.35	1.96	0.56	1.41	1.00	1.38	1.38	2.64	1.27
ADIP8-7	1.27	0.63	0.46	0.73	1.02	1.40	0.47	1.52	0.88	1.29	1.97	2.64	1.19
ADIP8-10	1.27	0.60	0.48	0.73	1.42	1.55	0.45	1.09	0.81	1.38	1.06	1.03	0.99
average	1.22	0.83	1.02	0.86	1.97	2.25	0.83	2.21	0.83	1.13	1.79	2.06	1.42
# >wt	0	17	14	18	4	1	16	4	20	9	7	4	
% >wt	0.0	70.8	58.3	75.0	16.7	4.2	66.7	16.7	83.3	37.5	29.2	16.7	

Additionally, each evolved isolate was tested for cross-tolerance toward other dicarboxylic acids of interest. First, K-12 MG1655 was tested in the Growth Profiler screening format for growth in the presence of a range of concentrations of each compound (note that this had been done in the Biolector format previously for pimelic acid and sebacic acid (Table 7) thus was not repeated here): fumaric acid, itaconic acid, malic acid, and succinic acid. All diacids tested were either the neutral sodium salts, or the free diacid was neutralized with sodium hydroxide to pH 7.0 for testing. Variable concentrations of these compounds elicited growth inhibition in *E. coli* K-12 MG1655 (Table 25). Based on these results, a screening

concentration was selected for the evolved isolates for which wild-type cells could achieve at a growth rate of 0.15-0.3 h^{-1} (versus uninhibited growth at 0.7-0.9 h^{-1} in M9 glucose minimal medium). These concentrations were: 45 g/L fumaric acid, 45 g/L itaconic acid, 55 g/L malic acid, 50 g/L succinic acid, 45 g/L pimelic acid, and 38 g/L sebacic acid. The results of glutaric acid and adipic acid-evolved isolates grown in these concentrations of fumaric, itaconic, and malic acid are shown in are shown in Tables 26 and 27, and the same isolates grown at the selected concentration of succinic, pimelic, and sebacic acid (linear aliphatic diacids) are shown in Tables 28 and 29. A majority of evolved isolates exhibited increased growth rates and/or reductions in lag time in all tested diacids. These in particular included isolates from the ADIP1, ADIP2, ADIP3, and ADIP7 populations, ADIP5-6 (a hypermutator strain) for all diacids generally. ADIP7-2 and ADIP7-5 exhibited the highest growth rates in sebacic acid, with the ADIP2 population also exhibiting significantly improved growth. ADIP7-2 and ADIP7-5 possessed different sets of mutations but notably only possessed loss-of-function mutations in *kgtP* plus distinct modulatory mutations (probable reduction-of-function of *rpoS* based on the Keio collection screen and loss-of-function of *pstS* plus an intergenic insertion between *hns* and *tdk* in ADIP7-2, and loss-of-function of *ybjL, proV,* and probable reduction-of-function of *sspA* based on the Keio collection screen in ADIP7.5). The common features of ADIP2 isolates were possessing only loss-of-function mutations in *kgtP*, deletion of *proVWX,* and mutations that restore expression of PyrE. Glutaric acid evolved isolates tended to exhibit a specificity toward tolerance to particular diacids. Isolates from the GLUT5 population exhibited significantly improved growth rates in fumaric acid (these isolates exhibited probable reduction-of-function of *sspA* and the RpoB-K203T and SpoT-R236L mutations), whereas the GLUT1 and GLUT2 populations had some of the most improved growth rates and reduced lag times in itaconic acid (these isolates featured loss-of-function of *kgtP* and the SpoT-V422A and RpoC-H419P mutations in GLUT1-3 and GLUT1-9; loss-of-function of *kgtP, proV,* probable reduction-of-function of *sspA*, and the SpoT-V422A mutation in GLUT1-10; loss-of-function of *kgtP* and optionally *proV,* and the SpoT-A451D mutation in GLUT2-1 and GLUT2-9; and loss-of-function of *kgtP* and *proV* plus the SpoT-A451D and RpoC-H419P mutations in GLUT2-10) . Malic acid cross-tolerance was weak across all glutaric acid evolved isolates. Isolates from the GLUT8 population had dramatically improved growth rates (as well as moderate reductions in lag time) toward sebacic acid (these isolates commonly featured loss-of-function of *kgtP* and *proV*, and the RpoC-P64L and PolB-R477G mutations, with the best performing isolates GLUT8-6 and GLUT8-9 additionally possessing the SapC-G79W mutation), with GLUT1-10 and GLUT2-9 also exhibiting significantly enhanced growth rates. Notably, the GLUT8 population had relatively poor cross-tolerance for most diacids, and all tested isolates featured the PolB-R477G mutation, whereas all other populations featured isolates with mutations in SpoT.

Table 25. *Growth rates and lag times of K-12 MG1655 in varying concentrations of the sodium salts of fumaric, itaconic, malic, and succinic acid, as measured in the Growth Profiler testing format.*

	fumaric acid				itaconic acid				malic acid				succinic acid			
	mean		std. error		mean		std. error		mean		std. error		mean		std. error	
diacid (g/L)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.747	5.1	0.030	0.2	0.747	5.1	0.030	0.2	0.747	5.1	0.030	0.2	0.747	5.1	0.030	0.2
10	0.662	5.8	0.021	0.0	0.709	5.8	0.037	0.1	0.686	5.9	0.053	0.0	0.665	5.4	0.084	0.0
20	0.615	6.9	0.019	0.0	0.603	7.0	0.036	0.2	0.639	6.6	0.024	0.1	0.610	6.7	0.024	0.1
30	0.482	9.5	0.009	0.1	0.520	9.4	0.012	0.2	0.526	8.0	0.002	0.2	0.496	8.9	0.028	0.1
40	0.285	15.4	0.005	0.2	0.320	14.1	0.014	0.2	0.410	11.1	0.005	0.2	0.345	13.4	0.014	0.1
50	0.116	30.2	0.011	1.0	0.117	29.9	0.008	0.7	0.287	16.9	0.002	0.2	0.179	22.4	0.008	0.0
60	-	-	-	-	-	-	-	-	0.154	28.3	0.006	0.3	0.127	42.5	0.016	1.6

Table 26. *Growth rates and lag times of K-12 MG1655 and glutaric acid-evolved isolates in specified inhibitory concentrations of fumaric, itaconic, and malic acid (sodium salts or neutralized with sodium hydroxide to pH 7.0), as measured in the Growth Profiler testing format.*

	45 g/L fumaric acid				45 g/L itaconic acid				55 g/L malic acid			
	mean		std. error		mean		std. error		mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.216	16.6	0.017	0.3	0.248	13.4	0.014	0.4	0.235	17.1	0.008	0.3
GLUT1-3	0.293	13.3	0.019	1.5	0.454	10.2	0.039	1.2	0.294	13.5	0.044	1.5
GLUT1-9	0.274	14.9	0.028	1.2	0.446	10.4	0.028	0.4	0.259	15.3	0.019	0.9
GLUT1-10	0.289	18.9	0.015	7.2	0.446	18.1	0.028	13.7	0.286	19.2	0.027	8.0
GLUT2-1	0.277	13.5	0.023	0.7	0.385	10.8	0.036	0.3	0.273	13.7	0.025	0.4
GLUT2-9	0.267	12.2	0.007	0.4	0.415	9.0	0.028	0.3	0.270	12.5	0.021	0.4
GLUT2-10	0.312	12.3	0.013	1.0	0.494	8.8	0.030	0.4	0.282	12.2	0.038	0.9
GLUT3-5	0.263	16.6	0.014	3.3	0.310	12.0	0.058	2.0	0.257	15.4	0.014	2.3
GLUT3-7	0.264	16.2	0.023	3.3	0.326	11.7	0.017	1.7	0.234	15.5	0.053	3.5
GLUT3-9	0.305	24.3	0.012	0.7	0.257	14.5	0.094	1.9	0.252	20.5	0.015	1.5
GLUT4-1	0.295	14.6	0.026	0.7	0.414	11.7	0.016	0.9	0.264	15.9	0.014	0.7
GLUT4-4	0.287	15.3	0.015	0.4	0.339	13.4	0.110	1.6	0.264	16.5	0.012	0.2
GLUT4-10	0.274	16.3	0.014	1.6	0.328	13.5	0.016	3.2	0.250	15.6	0.017	1.5
GLUT5-4	0.386	14.1	0.016	1.5	0.398	15.2	0.056	6.4	0.272	15.3	0.022	1.7
GLUT5-5	0.375	15.1	0.008	0.2	0.368	11.4	0.078	0.8	0.275	16.6	0.032	0.5
GLUT5-9	0.370	14.9	0.013	0.6	0.438	22.5	0.026	2.8	0.246	16.4	0.026	0.9
GLUT6-4	0.281	13.8	0.020	0.6	0.360	11.3	0.080	1.0	0.251	14.5	0.022	0.7
GLUT6-5	0.279	13.2	0.007	0.4	0.420	10.6	0.007	0.5	0.241	13.8	0.014	0.8
GLUT6-10	0.277	14.2	0.018	0.8	0.502	10.5	0.150	0.7	0.245	14.8	0.071	1.8

GLUT7-2	0.304	14.6	0.008	0.6	0.441	10.6	0.025	0.2	0.275	16.1	0.035	1.4
GLUT7-6	0.261	19.5	0.007	1.1	0.293	13.7	0.015	0.3	0.247	21.9	0.015	5.5
GLUT7-7	0.193	22.0	0.076	2.9	0.239	8.5	0.207	7.4	0.185	19.1	0.022	1.0
GLUT8-5	0.247	14.4	0.024	0.6	0.372	17.3	0.024	8.5	0.180	15.8	0.034	0.9
GLUT8-6	0.272	43.5	0.107	13.5	0.236	10.3	0.206	9.2	0.208	32.7	0.047	3.7
GLUT8-9	0.196	54.3	0.033	9.0	0.224	12.0	0.195	10.4	0.192	47.7	0.052	15.3

Table 27. *Growth rates and lag times of K-12 MG1655 and adipic acid-evolved isolates in specified inhibitory concentrations of fumaric, itaconic, and malic acid (sodium salts or neutralized with sodium hydroxide to pH 7.0), as measured in the Growth Profiler testing format.*

	45 g/L fumaric acid				45 g/L itaconic acid				55 g/L malic acid			
	mean		std. error		mean		std. error		mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.212	15.5	0.012	0.8	0.257	13.0	0.016	0.4	0.222	16.5	0.031	1.6
ADIP1-1	0.347	10.2	0.017	0.0	0.466	8.3	0.019	0.2	0.333	12.8	0.028	1.6
ADIP1-9	0.328	9.8	0.001	0.5	0.434	8.7	0.007	0.2	0.336	10.7	0.015	1.0
ADIP2-5	0.358	11.4	0.040	0.3	0.440	8.3	0.028	0.3	0.338	17.9	0.027	1.1
ADIP2-6	0.380	11.0	0.006	0.3	0.470	8.2	0.009	0.1	0.348	16.8	0.007	1.1
ADIP2-10	0.353	11.0	0.014	0.6	0.438	8.3	0.015	0.4	0.347	16.8	0.017	1.8
ADIP3-2	0.327	9.4	0.011	1.1	0.459	7.9	0.004	0.9	0.318	10.7	0.016	2.1
ADIP3-4	0.324	9.7	0.002	0.6	0.451	8.4	0.018	0.5	0.347	9.9	0.009	0.9
ADIP3-8	0.343	10.1	0.008	1.3	0.433	8.5	0.023	1.1	0.329	11.4	0.007	2.0
ADIP4-1	0.264	11.9	0.005	0.4	0.339	11.2	0.023	0.6	0.275	12.1	0.006	0.6
ADIP4-8	0.254	12.4	0.009	1.2	0.326	10.4	0.029	1.1	0.238	13.4	0.016	2.1
ADIP5-2	0.280	10.6	0.074	3.5	0.354	9.4	0.108	2.7	0.299	10.1	0.052	2.1
ADIP5-6	0.444	9.0	0.017	1.0	0.527	7.2	0.020	0.6	0.404	10.2	0.018	2.4
ADIP6-3	0.275	11.3	0.066	1.2	0.327	9.9	0.088	0.9	0.295	11.6	0.057	1.6
ADIP6-9	0.250	12.3	0.047	2.2	0.337	10.3	0.057	1.7	0.250	12.7	0.073	2.7
ADIP6-10	0.308	10.0	0.026	0.2	0.343	9.1	0.059	0.6	0.323	10.0	0.026	0.6
ADIP7-2	0.312	10.1	0.011	0.2	0.342	7.9	0.122	1.2	0.329	10.3	0.006	0.3
ADIP7-5	0.326	10.0	0.010	0.7	0.402	8.6	0.012	0.4	0.326	10.2	0.016	0.6
ADIP8-3	0.202	14.4	0.003	0.3	0.308	11.7	0.006	0.1	0.194	14.4	0.038	0.5
ADIP8-7	0.188	16.1	0.040	2.4	0.235	12.5	0.138	1.2	0.202	14.9	0.003	0.3
ADIP8-10	0.221	14.3	0.015	0.5	0.290	11.3	0.025	0.3	0.202	14.2	0.006	0.4

Table 28. *Growth rates and lag times of K-12 MG1655 and glutaric acid-evolved isolates in specified inhibitory concentrations of succinic, pimelic, and sebacic acid (sodium salts or neutralized with sodium hydroxide to pH 7.0), as measured in the Growth Profiler testing format.*

	50 g/L succinic acid				45 g/L pimelic acid				38 g/L sebacic acid			
	mean		std. error		mean		std. error		mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.169	20.2	0.006	0.7	0.275	13.4	0.019	0.1	0.167	26.9	0.008	1.2
GLUT1-3	0.231	14.5	0.025	1.8	0.369	10.4	0.028	0.8	0.074	34.9	0.006	5.4
GLUT1-9	0.194	20.8	0.013	8.0	0.337	11.9	0.022	0.2	0.043	44.7	0.024	8.4
GLUT1-10	0.246	20.0	0.042	8.2	0.399	13.1	0.026	3.3	0.278	20.6	0.015	2.8
GLUT2-1	0.232	14.7	0.016	0.2	0.362	11.6	0.002	0.4	0.153	21.7	0.005	2.7
GLUT2-9	0.213	14.4	0.012	0.3	0.349	10.3	0.025	0.3	0.282	12.5	0.006	1.0
GLUT2-10	0.277	13.4	0.024	1.7	0.383	10.2	0.033	0.5	0.093	29.8	0.006	1.3
GLUT3-5	0.242	16.1	0.020	2.1	0.280	17.6	0.096	11.1	0.106	20.0	0.132	22.4
GLUT3-7	0.235	15.3	0.023	2.5	0.360	11.5	0.035	1.1	0.068	6.4	0.117	11.0
GLUT3-9	0.221	20.6	0.011	0.7	0.351	15.7	0.023	2.0	0.158	18.9	0.062	4.3
GLUT4-1	0.263	16.3	0.016	0.9	0.378	12.8	0.035	0.4	0.266	32.9	0.005	3.6
GLUT4-4	0.242	16.6	0.008	0.8	0.388	13.2	0.021	0.1	0.265	34.2	0.007	0.7
GLUT4-10	0.231	15.7	0.004	1.3	0.371	11.5	0.030	0.7	0.113	20.1	0.121	20.5
GLUT5-4	0.281	17.0	0.038	4.8	0.433	12.1	0.026	0.4	0.183	37.7	0.014	1.1
GLUT5-5	0.264	18.4	0.020	0.5	0.420	12.8	0.002	0.4	0.117	54.9	0.079	4.3
GLUT5-9	0.244	18.4	0.016	1.9	0.428	13.0	0.028	1.2	0.143	58.2	0.130	10.3
GLUT6-4	0.241	14.2	0.031	0.5	0.356	11.7	0.022	0.6	0.085	26.4	0.012	6.6
GLUT6-5	0.243	13.9	0.010	0.2	0.348	11.3	0.003	0.4	0.123	21.6	0.030	3.8
GLUT6-10	0.211	14.4	0.018	1.2	0.353	12.1	0.021	0.1	0.132	26.2	0.008	2.4
GLUT7-2	0.223	14.9	0.031	0.4	0.349	11.6	0.030	0.3	0.173	17.5	0.010	0.9
GLUT7-6	0.195	17.8	0.019	1.0	0.357	14.8	0.008	0.5	0.141	18.9	0.017	1.2
GLUT7-7	0.156	19.9	0.015	0.3	0.335	14.1	0.024	0.2	0.112	20.0	0.033	1.5
GLUT8-5	0.198	16.4	0.042	1.2	0.349	11.8	0.012	0.4	0.300	21.4	0.026	0.9
GLUT8-6	0.236	34.1	0.016	7.3	0.184	20.9	0.193	18.8	0.464	13.7	0.047	0.5
GLUT8-9	0.250	38.0	0.011	5.5	0.259	11.2	0.224	10.1	0.471	14.5	0.048	1.3

Table 29. *Growth rates and lag times of K-12 MG1655 and adipic acid-evolved isolates in specified inhibitory concentrations of succinic, pimelic, and sebacic acid (sodium salts or neutralized with sodium hydroxide to pH 7.0), as measured in the Growth Profiler testing format.*

	50 g/L succinic acid				45 g/L pimelic acid				38 g/L sebacic acid			
	mean		std. error		mean		std. error		mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.143	23.8	0.046	5.4	0.271	12.4	0.041	1.2	0.135	28.2	0.022	5.7
ADIP1-1	0.299	16.1	0.012	1.5	0.432	9.6	0.040	0.3	0.244	21.6	0.029	3.8

ADIP1-9	0.290	14.5	0.006	2.3	0.421	8.8	0.013	0.9	0.248	17.8	0.005	1.1
ADIP2-5	0.260	25.5	0.028	2.3	0.474	11.0	0.011	0.3	0.315	13.5	0.054	0.5
ADIP2-6	0.278	21.7	0.013	1.1	0.480	10.6	0.006	0.2	0.319	12.9	0.019	0.3
ADIP2-10	0.262	22.0	0.040	4.4	0.479	10.8	0.003	1.0	0.290	13.4	0.022	0.8
ADIP3-2	0.311	13.2	0.013	3.2	0.438	8.4	0.003	1.3	0.254	17.6	0.010	4.6
ADIP3-4	0.308	12.7	0.006	1.6	0.435	8.5	0.010	0.8	0.262	17.1	0.007	1.3
ADIP3-8	0.304	13.8	0.016	2.0	0.426	9.1	0.021	1.4	0.253	19.8	0.018	4.1
ADIP4-1	0.206	15.7	0.013	0.2	0.348	9.9	0.010	0.2	0.308	13.1	0.013	0.5
ADIP4-8	0.228	15.8	0.037	4.0	0.349	10.3	0.011	0.8	0.289	12.8	0.008	1.3
ADIP5-2	0.192	13.5	0.028	2.2	0.385	8.7	0.114	2.1	0.288	15.3	0.107	4.2
ADIP5-6	0.361	12.1	0.019	1.9	0.483	8.5	0.023	1.9	0.273	20.7	0.016	0.6
ADIP6-3	0.246	14.9	0.065	3.7	0.346	10.4	0.106	1.2	0.268	17.9	0.125	9.4
ADIP6-9	0.210	18.4	0.062	5.2	0.278	11.1	0.074	1.9	0.181	23.1	0.051	7.6
ADIP6-10	0.285	12.4	0.033	0.6	0.360	9.7	0.014	0.6	0.267	15.3	0.066	3.1
ADIP7-2	0.294	12.5	0.018	0.3	0.393	9.0	0.012	0.4	0.356	11.1	0.014	1.1
ADIP7-5	0.274	12.4	0.024	1.3	0.376	9.4	0.003	0.7	0.353	12.0	0.010	1.4
ADIP8-3	0.146	22.1	0.016	1.6	0.235	12.8	0.010	0.2	0.171	28.9	0.017	1.4
ADIP8-7	0.163	23.2	0.014	0.7	0.268	12.8	0.060	0.6	0.157	30.9	0.012	2.2
ADIP8-10	0.131	20.0	0.042	0.9	0.249	12.8	0.003	0.3	0.165	30.0	0.012	2.3

Biological production of diacids

Glutaric acid has been the target of two studies, both in engineered *E. coli*. The highest reported titer of 0.82 g/L from glucose was achieved via native L-lysine production (Adkins *et al.*, 2013) (see Figure 1 of Adkins *et al.*, 2013, hereby incorporated by reference). A heterologous pathway composed of genes from *Pseudomonas putida* KT2440 was expressed from plasmids and consisted of a lysine monooxygenase to convert lysine to 5-aminovaleramide, a 5-aminovaleramidase to convert 5-aminovaleramide to 5-aminovaleric acid, a 5-aminovalerate transaminase to convert 5-aminovaleric acid and α-ketoglutarate to glutarate semialdehyde and L-glutamate, and a glutarate semialdehyde dehydrogenase to convert glutarate semialdehyde to glutaric acid. To improve flux toward L-lysine, previously known feedback resistance mutations were made in DapA (4-hydroxytetrahydrodipicolinate synthase) and LysC (asparate kinase III), and these modified proteins were additionally overexpressed from plasmids. Finally, *cadA* and *ldcC*, encoding two lysine decarboxylases, were deleted to prevent side conversion of L-lysine into cadaverine. In a second study (Park *et al.*, 2013), glutarate was not able to be produced from glucose, however 1.7 g/L glutarate could be produced by feeding both L-lysine and α-ketoglutarate, with only 5-aminovalerate able to be produced from glucose without supplementation. This appeared to use the same or similar heterologous genes from *P. putida* as in the previous paper, only expressed together as an artificial operon on one plasmid instead of in two operons on two plasmids. The strain additionally contained a *dapA* promoter replacement to allow constitutive expression of lysine

biosynthesis, and deletion of *speE*, *speG*, *patA*, and *puuPA* (which would prevent production of spermidine, acetylspermidine, putrescine degradation, and putrescine import, although likely not for any targeted purpose here; use of this background strain for production of other compounds).

Overproduction of adipic acid, as well as other diacids that can be readily converted chemically into adipic acid, has been more heavily pursued due to the use of adipic acid in existing commercial polyamides. A wide variety of routes have been explored, with the first reported direct route in *E. coli* being a proof-of-concept demonstration, with a maximum titer of 639 µg/mL adipic acid (Yu *et al.*, 2014). In the best-performing strain, acetyl-CoA and succinyl-CoA were condensed by a reversible 3-oxoadipyl-CoA thiolase (PaaJ from *E. coli*), 3-oxoadipyl-CoA was reduced to 3-hydroxyadipyl-CoA by a 3-hydroxyacyl-CoA dehydrogenase (PaaH1 from *Ralstonia eutropha*), 3-hydroxyadipyl-CoA was dehydrated to 2,3-dehydroadipyl-CoA by by a putative enoyl-CoA hydratase (h16_AA307 gene product from *Ralstona eutropha* H16), 2,3-dehydroadipyl-CoA was reduced to adipyl-CoA by a *trans*-enoyl-CoA reductase (Ter from *Euglena gracilis*), adipyl-CoA was converted to adipyl-phosphate by a phosphate butyryltransferase (Ptb from *Clostridum acetobutylicum*), and adipyl-phosphate was finally dephosphorylated to adipic acid using a butyryl kinase (Buk1 from *Clostridium acetobutylicum*) (see Figure 1 of Yu *et al.*, 2014, hereby incorporated by reference). The genes encoding these enzymes were heterologously expressed on two different plasmids, and additional modifications were made to the genome to improve the succinyl-CoA supply using modifications that were previously employed for succinic acid production via succinyl-CoA (Liu *et al.*, *Process Biochem.* 47:1532, 2012). These were deletions of *ptsG*, *poxB, pta*, *sdhA*, and *iclR*.

Very recently, production of 2.5 g/L adipic acid in bioreactors, as well as smaller quantities of suberic acid (C_8) and sebacic acid (C_{10}), or pimelic acid (C_7) alone, was demonstrated in *E. coli* from glycerol using a relatively similar modular pathway (Cheong *et al.*, 2016). It was composed of a thiolase capable of condensing a primer and extender unit (*e.g.* succinyl-CoA and acetyl-CoA for adipic acid), plus a hydroxyacyl-CoA hydrogenase, an enoyl-CoA hydratase, an enoyl-CoA reductase to generate the fully reduced product (for adipic acid, this would be adipyl-CoA) (see Figure 1a of Cheong *et al.*, 2016, hereby incorporated by reference). The major difference from previous work was the use of an acyl-CoA thioesterase to liberate the final diacid from CoA. For production of adipic acid production, a CoA transferase (Cat1 from *Clostridium kluyveri*) was expressed for activation of succinic acid to succinyl-CoA, with native *sucD* encoding a subunit of native *E. coli* succinyl-CoA synthetase deleted. The thiolase was *E. coli* PaaJ, the hydroxyacyl-CoA hydrogenase was *E. coli* PaaH, the enoyl-CoA hydratase was *E. coli* PaaF, the enoyl-CoA reductase was Ter from *Treponema denticola*, and the acyl-CoA thioesterase was the dicarboxylic acyl-CoA thioesterase Acot8

from *Mus musculus*. Additionally, fermentative pathways leading to production of acetate (*pta* and *poxB*), lactate (*ldhA*), and ethanol (*adhE*) were deleted from the background strain. To produce approximately 25 mg/L pimelic acid, glutaric acid was fed to generate glutaryl-CoA as a primer unit via the action of Cat1 in a background strain deficient in *ldhA*, *poxB, pta, adhE,* and the gene encoding fumarate reductase, *frdA*. To generate a mixture containing predominantly adipic acid at 95 mg/L, but also 34 mg/L suberic acid and 13 mg/L sebacic acid, an alternative thiolase (DcaF), hydroxyacyl-CoA dehydrogenase (DcaH), and enoyl-CoA hydratase (DcaE) from *Acinetobacter sp.* ADP1, with other enzymes the same as for producing adipic acid. In this case, the background strain was the same as that used for pimelic acid, with additional deletions in a number of other native *E. coli* acyl-CoA thioesterases (*yciA*, *ybgC*, *ydiI*, *tesA*, *fadM*, and *tesB*).

An alternative native adipate production pathway (reverse adipate degradation) has been reported in the bacterium *Thermobifida fusca* B6, where succinyl-CoA and acetyl-CoA are condensed by a β-ketothioase (EC 2.3.1.174) to form 3-oxoadipyl-CoA, followed by a series of reactions that are the same as those shown in Figure 1 of Yu *et al.*, 2014, to form adipyl-CoA. Adipyl-CoA is subsequently converted to adipic acid by a succinyl-CoA synthetase (Tfu_2577, Tfu_2576). A titer of over 2 g/L of adipic acid was obtained by fermentation of *T. fusca* B6 on glucose and milled corncob (Deng and Mao, 2015). The pathway has not yet been heterologously expressed in other organisms.

Adipic acid production from *S. cerevisiae* has been described by Verdezyne (*e.g.* WO 2011/003034 A2), however the starting substrates are fatty acids and the pathway for adipic acid production is therefore very different. The engineered microorganisms described have genetic modifications that add or increase the 6-oxohexanoic acid dehydrogenase, omega oxo fatty acid dehydrogenase, 6-hydroxyhexanoic acid dehydrogenase, omega hydroxyl fatty acid dehydrogenase, hexanoate synthase, monooxygenase, monooxygenase reductase, fatty alcohol oxidase, acyl-CoA ligase, acyl-CoA oxidase, enoyl-CoA hydratase, 3-L-hydroxyacyl-CoA dehydrogenase, and/or acetyl-CoA C-acetyltransferase activities. These modifications suggest a pathway where a fatty acid is broken down into multiple shorter chain diacids, derivatives are condensed with acetyl-CoA to increase the chain length where necessary, and C_6 diacid products are reduced to adipic acid following some similar steps to those shown in Figure 1a of Cheong *et al.*, *supra*).

In addition to directly producing the final diacid products, other groups have developed alternative pathways to *cis,cis*-muconic acid, which can be chemically or enzymatically reduced to adipic acid, or glucaric acid, which can be produced in few steps from glucose and can be chemically reduced to adipic acid. Basic pathway schematics are shown in Figure 4 of

Polen *et al.* (2013) for muconic acid, and Figure 5 of Polen *et al.* (2013) for glucaric acid. Figures 4 and 5 of Polen *et al.* (2013) are hereby incorporated by reference.

Muconic acid has been produced at 141 mg/L from glucose in *S. cerevisiae* (Curran *et al.*, 2013) in a strain possessing a deletion in ARO3 (a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase isoform), expression of a feedback resistant version of ARO4 (another 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase isoform; the modifications included both a feedback resistant coding mutation and a constitutive promoter replacement), and a deletion in ZWF1 (glucose-6-phosphate dehydrogenase). The heterologous pathway was expressed on 3 plasmids containing codon-optimized DHS from *Podospora anserina*, catechol 1,2-dioxygenase from *Candida albicans*, overexpressed TKL1 (transketolase) from *S. cerevisiae*, and protocatechuate decarboxylase from *Enterobacter cloacae*. To obtain higher expression of protocatechuate decarboxylase, another copy of the gene was also integrated onto the chromosome. The combination of non-pathway mutations (deletion of ZWF1 and ARO3; expression of feedback-resistant ARO4; overexpression of TKL1) served to relieve feedback inhibition of the shikimate pathway that is ordinarily employed for aromatic amino acid biosynthesis, and to direct flux into the pentose phosphate pathway via transketolase instead of glucose-6-phosphate dehydrogenase, increasing the supply of the erythrose-4-phosphate precursor.

Muconic acid has additionally been produced (170 mg/L of muconic acid) from glucose in *E. coli* possessing deletions in *ptsH*, *ptsI*, *crr*, and *pykF* and overexpressing *ubiC*, a feedback resistant version of *aroF*, *aroE*, and *aroL* (Sengupta *et al.*, 2015). These mutations increase the supply of the precursors erythrose-4-phosphate and phosphoenolpyruvate. The heterologous pathway was composed of *pobA* from *Pseudomonas putida* KT2440, *aroY* from *Klebsiella pneumoniae*, and *catA* from *Acinetobacter sp.* strain ADP1. *E. coli* co-cultures consisting of strains engineered to overproduce DHS, and to convert DHS to muconic acid, have additionally been engineered to produce muconic acid from glycerol at a final titer of 2 g/L (Zhang *et al.*, *Microb. Cell Factories* 14:134, 2015).

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WO 2016/162442 A1 (Metabolic Explorer SA)

CLAIMS

1. A bacterial cell comprising a biosynthetic pathway for producing an aliphatic dicarboxylic acid and at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *kgtP, ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG*, and *yeaR*, or a combination of any thereof.

2. The bacterial cell of claim 1, comprising a genetic modification which reduces the expression of *kgtP*.

3. The bacterial cell of any one of the preceding claims, comprising at least one genetic modification which reduces the expression of *ybjL, proV*, *proW, proX, sspA* or a combination of any thereof.

4. The bacterial cell of any one of the preceding claims, comprising genetic modifications which reduce the expression of

(a) *kgtP* and at least one of *proV, proW* and *proX*;
(b) *kgtP* and *ybjL;*
(c) *kgtP*, *ybjL*, and at least one of *proV, proW* and *proX*;
(d) *kgtP*, *ybjL*, and at least one of *nagA* and *nagC*;
(e) *kgtP, ybjL,* at least one of *nagA* and *nagC,* and at least one of *proV, proW* and *proX*;
(f) *kgtP* and *sspA*; or
(g) *kgtP*, *tdk* and *pstS.*

5. The bacterial cell of any one of the preceding claims, wherein the genetic modification comprises a knock-down or knock-out of the endogenous gene or genes.

6. The bacterial cell of any one of the preceding claims, wherein the genetic modification provides for an increased growth rate, a reduced lag time, or both, of the cell in the presence of at least one of glutaric acid and adipic acid as compared to the bacterial cell without the genetic modification.

7. A bacterial cell genetically modified from a parent bacterial cell so as to comprise

(a) a mutant SpoT, comprising at least one mutation in the threonyl-tRNA synthetase GTPase and SpoT (TGS) domain corresponding to amino acid

residues I388 to T447 and/or the linker segment between the TGS and the aspartokinase, chorismate mutase and TyrA (ACT) domain corresponding to amino acid residues A448 to T621, optionally in one or more amino acid residues selected from A451, R236, V422, W457, N454, D580, M247, T442, S434, N601, I602 and R603;

(b) a mutant PolB, comprising a mutation in amino acid residue R477;

(c) a mutant RpoC, comprising a mutation in at least one of the amino acid residues corresponding to H419 and P64;

(d) a mutant RpoB, comprising a mutation in an amino acid residue corresponding to K203;

(e) a mutant Rnt, comprising a mutation in at least one of the amino acid residues corresponding to Q179, A27, F194 and A180;

(f) a mutant SapC, comprising a mutation in the amino acid residue corresponding to G79;

(g) increased expression of PyrE as compared to the parent bacterial cell; or

(h) a combination of any two or more of (a) to (g),

optionally in combination with a knock-down or knock-out of the endogenous gene or genes according to claim 5;

wherein the genetic modification provides for an increased growth rate, a reduced lag time, or both, in the presence of at least one of glutaric acid and adipic acid as compared to the parent bacterial cell.

8. The bacterial cell of claim 7, comprising

(a) at least one mutant protein selected from the group consisting of SpoT-V422A, SpoT-A451D, SpoT-A451V, SpoT-W457C, SpoT-N454H, SpoT-D580Y, SpoT-R236L, SpoT-R236S, SpoT-M247K, SpoT-NIR(601-603)S, SpoT-T442I, SpoT-S434L, PolB-R477G, RpoC-H419P, RpoC-P64L, RpoB-K203T, Rnt-Q179P, Rnt-A27T, Rnt-F194L, Rnt-A180T, SapC-G79W; and/or

(b) a mutation in *rph* or the pyrE/rph intergenic region which increases the expression of PyrE.

9. The bacterial cell of any one of the preceding claims, comprising a recombinant biosynthetic pathway for producing at least one of glutaric acid, adipic acid, succinic acid, muconic acid, fumaric acid, itaconic acid, malic acid, malonic acid, maleic acid, glucaric acid, pimelic acid, suberic acid, sebacic acid, 2,5-furandicarboxylic acid, terephthalic acid, or azelaic acid, mesaconic acid, citraconic acid, tartaric acid, tartronic acid, diaminopimelic acid and glutaconic acid.

10. A process for preparing a recombinant bacterial cell for producing an aliphatic dicarboxylic acid, the process comprising genetically modifying an *E. coli* cell to

(a) introduce a recombinant biosynthetic pathway for producing the aliphatic dicarboxylic acid, and

(b) knock-down or knock-out of the endogenous gene or genes according to claim 5, and/or

(c) express a mutant of at least one of SpoT, PolB, RpoC, RpoB, Rnt and SapC and/or increase expression of PyrE according to claim 7;

wherein steps (a), (b) and (c) are performed in any order.

11. A process for improving the tolerance of a bacterial cell to an aliphatic dicarboxylic acid comprising genetically modifying the bacterial cell to

(a) knock-down or knock-out of the endogenous gene or genes according to claim 5; and/or

(b) express a mutant of at least one of SpoT, PolB, RpoC, RpoB, Rnt and SapC and/or increase expression of PyrE according to claim 7;

wherein steps (a) and (b) are performed in any order.

12. The bacterial cell of any one of claims 1 to 10 or the process of any one of claims 11 and 12, wherein the bacterial cell is of the *Escherichia, Lactobacillus, Lactococcus, Bacillus, Pseudomonas, Corynebacterium, Deinococcus* or *Ralstonia* species, such as of the *Escherichia coli* species.

13. A method for producing an aliphatic dicarboxylic acid, comprising culturing the bacterial cell of any one of claims 1 to 10 and 13 or the bacterial cell obtained by the process of any one of claims 11 to 13, in the presence of a carbon source, and, optionally, isolating the aliphatic dicarboxylic acid.

14. A composition comprising glutaric acid or adipic acid at a concentration of at least 5 g/L and a plurality of bacterial cells of the *Escherichia* genus genetically modified to

(a) knock-down or knock-out of the endogenous gene or genes according to claim 5; and/or

(b) comprise a mutant of at least one of SpoT, PolB, RpoC, RpoB, Rnt and SapC and/or increased expression of PyrE according to claim 7.

15. The bacterial cell of any one of claims 1 to 10 and 13, or the process according to any one of claims 11 to 13, wherein the biosynthetic pathway for producing an aliphatic dicarboxylic acid comprises

(a) a lysine monooxygenase, a 5-aminovaleramidase, a 5-aminovalerate transaminase, and a glutaraldehyde semialdehyde dehydrogenase;

(b) a reversible 3-oxoadipyl-CoA thiolase, a 3-hydroxyacyl-CoA dehydrogenase, an enoyl-CoA hydratase, an enoyl-CoA reductase, and either a terminal carboxyacyl-CoA thioesterase, or a terminal carboxyacyl-CoA phosphotransferase and a reversible alkyl-1,n-dicarboxylate kinase, where n is the carbon chain length of the product; and, optionally a malonyl-CoA or glutaryl-CoA transferase; or

(c) a 2-dehydro-3-deoxy-D-arabinoheptonate-7-phosphate synthase, a 3-dehydroquinate synthase, a 3-dehydroxyquinate dehydratase, a dehydroshikimic acid dehydratase, a protocatechuate decarboxylase, and a catechol 1,2-dioxygenase.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/079313

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20 C12P7/44 C12P7/46
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DAVID BYRNE ET AL: "Comparative multi-goal tradeoffs in systems engineering of microbial metabolism", BMC SYSTEMS BIOLOGY, BIOMED CENTRAL LTD, LO, vol. 6, no. 1, 26 September 2012 (2012-09-26), page 127, XP021107952, ISSN: 1752-0509, DOI: 10.1186/1752-0509-6-127	1,2,5,6, 11-13
Y	table 1	3,4,9, 10,14,15

X	WO 01/05959 A1 (AJINOMOTO KK [JP]; KIMURA EIICHIRO [JP]; ITO HISAO [JP]; KURAHASHI OSA) 25 January 2001 (2001-01-25)	1,2,5,6, 11-13
Y	claims 1,6; table 2	9,10,14

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 January 2018	15/03/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bucka, Alexander

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2004/033421 A2 (GENENCOR INT [US]; CERVIN MARGUERITE A [US]; SOUCAILLE PHILIPPE [FR];) 22 April 2004 (2004-04-22) claims 1,5,8	1-6,9-15
Y	DENG YU ET AL: "Biological production of adipic acid from renewable substrates: Current and future methods", BIOCHEMICAL ENGINEERING JOURNAL, vol. 105, 28 August 2015 (2015-08-28), pages 16-26, XP029290467, ISSN: 1369-703X, DOI: 10.1016/J.BEJ.2015.08.015	9,10,14, 15
A	page 24, left-hand column	1-6,11, 12
A	LEE J-K ET AL: "Characteristics of acid tolerance of adipic acid-resistant mutant strain, Leuconostoc mesenteroides", SANNEB MISAINMURHAG HOIJI - KOREAN JOURNAL OF APPLIED MICROBIOLOGY AND BIOENGINEERING, SEOUL, KR, vol. 28, no. 2, April 2000 (2000-04), pages 63-70, XP009106562, ISSN: 0257-2389 abstract	1-6,9-15
X	WO 2016/162442 A1 (METABOLIC EXPLORER SA [FR]) 13 October 2016 (2016-10-13) example 3	1,2,5,6, 11-13
Y	N. DOUKYU ET AL: "Improvement in organic solvent tolerance by double disruptions of proV and marR genes in Escherichia coli", JOURNAL OF APPLIED MICROBIOLOGY., vol. 112, no. 3, 31 January 2012 (2012-01-31), pages 464-474, XP055356725, GB ISSN: 1364-5072, DOI: 10.1111/j.1365-2672.2012.05236.x cited in the application figure 1; table 1	3,4
Y	JAKE ADKINS ET AL: "Engineering Escherichia coli for renewable production of the 5-carbon polyamide building-blocks 5-aminovalerate and glutarate", BIOTECHNOLOGY AND BIOENGINEERING, vol. 110, no. 6, 17 January 2013 (2013-01-17), pages 1726-1734, XP055358169, ISSN: 0006-3592, DOI: 10.1002/bit.24828 cited in the application figure 1	15

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W. GUO ET AL: "Ketoglutarate Transport Protein KgtP Is Secreted through the Type III Secretion System and Contributes to Virulence in Xanthomonas oryzae pv. oryzae", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 78, no. 16, 8 June 2012 (2012-06-08), pages 5672-5681, XP055355965, US ISSN: 0099-2240, DOI: 10.1128/AEM.07997-11 figures 1,2	1,2,5,6, 13
X	KOITA K ET AL: "Identification and analysis of the putative pentose sugar efflux transporters in Escherichia coli", PLOS ONE, PUBLIC LIBRARY OF SCIENCE, US, vol. 7, no. 8, 28 August 2012 (2012-08-28) , pages e43700-1, XP002753123, ISSN: 1932-6203, DOI: 10.1371/JOURNAL.PONE.0043700 [retrieved on 2012-08-28] table 3	1,2,5,6, 13
Y	SUDESHNA SENGUPTA ET AL: "Metabolic Engineering of a Novel Muconic Acid Biosynthesis Pathway via 4-Hydroxybenzoic Acid in Escherichia coli", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 81, no. 23, 11 September 2015 (2015-09-11), pages 8037-8043, XP055358177, US ISSN: 0099-2240, DOI: 10.1128/AEM.01386-15 cited in the application figure 1	15
Y	JIA-LE YU ET AL: "Direct biosynthesis of adipic acid from a synthetic pathway in recombinant Escherichia coli", BIOTECHNOLOGY AND BIOENGINEERING, vol. 111, no. 12, 3 June 2014 (2014-06-03) , pages 2580-2586, XP055357660, ISSN: 0006-3592, DOI: 10.1002/bit.25293 cited in the application figure 1	15

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. D. WINKLER ET AL: "Evolved Osmotolerant Escherichia coli Mutants Frequently Exhibit Defective N-Acetylglucosamine Catabolism and Point Mutations in Cell Shape-Regulating Protein MreB", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 80, no. 12, 11 April 2014 (2014-04-11), pages 3729-3740, XP055356724, US ISSN: 0099-2240, DOI: 10.1128/AEM.00499-14 table 1	3,4
A	EP 2 147 970 A1 (AJINOMOTO KK [JP]) 27 January 2010 (2010-01-27) claims 1,9	3,4
A	ANNE-MARIE HANSEN ET AL: "SspA is required for acid resistance in stationary phase by downregulation of H-NS in Escherichia coli", MOLECULAR MICROBIOLOGY., vol. 56, no. 3, 15 March 2005 (2005-03-15) , pages 719-734, XP055356726, GB ISSN: 0950-382X, DOI: 10.1111/j.1365-2958.2005.04567.x figure 3	3,4
A	TINO POLEN ET AL: "Toward biotechnological production of adipic acid and precursors from biorenewables", JOURNAL OF BIOTECHNOLOGY, vol. 167, no. 2, 21 July 2012 (2012-07-21) , pages 75-84, XP055119139, ISSN: 0168-1656, DOI: 10.1016/j.jbiotec.2012.07.008 cited in the application the whole document	1-6,9-15
A	JAMES B MCKINLAY ET AL: "Prospects for a bio-based succinate industry", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 76, no. 4, 4 July 2007 (2007-07-04), pages 727-740, XP019538718, ISSN: 1432-0614, DOI: 10.1007/S00253-007-1057-Y table 1	1-6,9-15

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/079313

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-6, 9-15(all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-6, 9-15(all partially)

A bacterial cell comprising a biosynthetic pathway for producing an aliphatic dicarboxylic acid and at least one genetic modification which reduces expression of the endogenous kgtP gene.

2-21. claims: 1-6, 9-15(all partially)

A bacterial cell comprising a biosynthetic pathway for producing an aliphatic dicarboxylic acid and at least one genetic modification, wherein in each separate invention the modification reduces expression of an endogenous gene selected from ybjL, proV, proW, proX, proQ, cspE, rfaE, yfbP, yfjM, pstS, pstA, pstB, pstC, rph, rpoS, sspA, tdk, uvrB, ycjG, and yeaR, or a combination of any thereof.

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22. claims: 7-15(partially)

A bacterial cell genetically modified from a parent bacterial cell so as to comprise(a) a mutant SpoT, comprising at least one mutation in the threonyl-tRNA synthetase GTPase and SpoT (TGS) domain corresponding to amino acid residues 1388 to T447 and/or the linker segment between the TGS and the aspartokinase, chorismate mutase and TyrA (ACT) domain corresponding to amino acid residues A448 to T621, optionally in one or more amino acid residues selected from A451, R236, V422, W457, N454, D580, M247, T442, S434, N601, 1602 and R603.

23-28. claims: 7-15(partially)

A bacterial cell genetically modified from a parent bacterial cell so as to comprise a modification, wherein in each separate invention the modification is one of (b) a mutant PolB, comprising a mutation in amino acid residue R477; (c) a mutant RpoC, comprising a mutation in at least one of the amino acid residues corresponding to H419 and P64; (d) a mutant RpoB, comprising a mutation in an amino acid residue corresponding to K203; (e) a mutant Rnt, comprising a mutation in at least one of the amino acid residues corresponding to Q179, A27, F194 and A180; (f) a mutant SapC, comprising a mutation in the amino acid residue corresponding to G79; (g) increased expression of PyrE as compared to the parent bacterial cell.

INTERNATIONAL SEARCH REPORT

Information on patent family members

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